**Comparative Cellular and Molecular Responses of
Insect and Human Cells Exposed to Commercial
Bacillus thuringiensis Bioinsecticides**

by

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A thesis submitted to the Faculty of Graduate Studies in
partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Biology Department, **CARLETON UNIVERSITY,**

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in collaboration with

Health Canada, Mutagenesis Section,

Environmental Health Centre

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Bacillus thuringiensis Bioinsecticides**

Submitted by

Azam Tayabali, B.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

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Abstract

This thesis describes a detailed *in vitro* toxicological analysis of commercial *Bacillus thuringiensis*-based biopesticides (BT) and subcomponents, using bioassays involving insect and human cell lines as targets. The BT products are concentrated cultures, containing *B.t. kurstaki* (*Btk*) or *B.t. israelensis* (*Bti*) spores ($>10^9/\text{mL}$), variable amounts of cellular debris and intact parasporal inclusion bodies (PIBs), and polypeptides mainly related to δ -endotoxins. Simulated field doses of 10^{-7} to 10^{-3} International Units per target cell were used to study responses in: morphology, monitored by phase contrast and electron microscopy; bio-reduction with reporter dyes; protein levels and synthesis, measured by immunoprobes and ^{35}S -methionine incorporation, and DNA integrity. With antibiotic to inhibit spore activity, cell changes were affected by only BT doses $>10^{-3}$ I.U./cell. Without antibiotic, BT exhibited dose-lag / dose-dependent damage caused by vegetative *Bt* cells, resulting in target cell shedding, loss in bio-reduction, protein markers and DNA integrity, and appearance of nuclear pyknosis and cytolysis. BT dose-induced damage of insect and human cell types was remarkably similar, differing only in response time due to assay temperature (27°C versus 37°C) which affected *Btk* or *Bti* proliferation rate. Equivalent exposures with *Bacillus subtilis* and *Escherichia coli* produced minor effects. Cytotoxicity of BT supernatants free of spores and PIBs was seen in 2-h bioassays at doses equivalent to ≥ 1 I.U. / cell. This activity was $\leq 0.08\%$ of that of vegetative cell culture filtrates (CF) derived from BT products (spores). ^{35}S -methionine labelling of target cells showed that CF-exposure rapidly arrested protein synthesis. Studies with intact PIBs, proteins derived from them (activated δ -endotoxins), *Bt* phosphatidylinositol phospholipase C, and *Bacillus cereus* enterotoxin, all

showed that CF was the most toxic agent. This CF toxicity could be abolished by pre-treatment with a broad-spectrum protease or heat $\geq 60^{\circ}\text{C}$. Column chromatography of CF gave several activity peaks, but ^{32}P -NAD-mediated ribosylation revealed only one product of 45 kDa. These results substantiate that toxification of BT products can be practically compared and quantified by various bioindicators *in vitro*, and that the most toxic effect arises as an outcome of infection of either insect or human cells by even a single spore ($\sim 10^{-9}$ to 10^{-10} I.U./cell). The finding that vegetative cells of *Btk* and *Bti* produce the most potent cytotoxins of all BT product subcomponents questions simplistic views on mode of action attributed to existing BT products when used in environmental applications.

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List of Abbreviations

AGE	agarose gel electrophoresis
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCET	<i>Bacillus cereus</i> enterotoxin
bp	base pair(s)
BT	commercial formulation derived from <i>Bacillus thuringiensis</i> subspecies
<i>Bti</i>	<i>Bacillus thuringiensis</i> subsp. <i>israelensis</i>
<i>Btk</i>	<i>Bacillus thuringiensis</i> subsp. <i>kurstaki</i>
°C	degrees Celsius
Caco-2	human colon adenocarcinoma cell line
CAM(s)	cell adhesion molecules
CF	CF
cfu	colony-forming unit
DP	commercial BT product, Dipel
DNA	deoxyribonucleic acid
DNP	dinitrophenol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
EDTA	N,N-ethylenediaminetetraacetic acid
F48	commercial BT product, Foray 48B®
F76	commercial BT product, Foray 76B®
x G	times gravity
GST	glutathione-S-transferase
h	hour(s)
Hep-G2	human hepatoblastoma cell line
HL-60	human promyelocytic leukaemia cell line
hsp 60 or hsp 70	heat shock protein subfamilies of either 60 or 70 kDa
HT29	human colon adenocarcinoma cell line
ID ₅₀	dose necessary to cause 50% inhibition (inhibitory dose)
I.U.	International Units of BT Product Potency
K562	human myelogenous leukaemia cell line
kDa	kilodalton(s)
µg or mg or g	microgram(s) or milligram(s) or gram(s)
µm or mm	micrometre or millimetre
µM or mM	micromolar or millimolar
µL or mL or L	microlitre(s) or millilitre(s) or litre(s)
LB	Luria Bertani broth
MG1	cell line derived from midgut tissue of a larval cabbage looper, <i>Trichoplusia ni</i>
min	minute(s)

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaN ₃	sodium azide
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIB	parasporal inclusion body
PI-PLC	phosphatidylinositol phospholipase C
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
Sy9	cell line from ovaries of fall armyworm, <i>Spodoptera frugiperda</i>
TH	commercial BT product, Thuricide
TBS	tris-buffered saline
TN-368	cell line from ovary of <i>Trichoplusia ni</i>
TEM	transmission electron microscopy
Vb	commercial BT product, VectoBac 12AS®
VIP	Vegetative Insecticidal Protein(s)
v/v	volume per volume
w/v	weight per volume
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide sodium salt

1. INTRODUCTION

1.1 General Scope of Thesis

Higher eukaryotes (eg. insects, mammals and plants) coexist with a vastly larger population of diverse organisms collectively called microbes. The latter include viruses, bacteria, and lower eukaryotes such as yeast, fungi, protozoa and nematodes. A relatively small proportion of these microbes are known to be pathogenic in the sense that they can cause host-related cell, tissue or organ malfunctions, leading in some cases to death. What defines the ability of a microbe to be pathogenic depends on its peculiar physical-biochemical properties, often considered as virulence factors, and the extent to which evasive or “defensive” mechanisms of the host, target organism are effective. If the host defence mechanisms are absent or are in some way compromised, even the least opportunistic microbes can take advantage of favourable situations causing infection and disease. The schematic in Figure 1 shows some of the interactive mechanisms of microbes and animal cells. Here, it is recognized that microbes are produced “naturally” or “industrially” as biotechnology products for environmental use. The major differences between the latter and the former, are the quality and quantity of specific microbes, and the additives that may be present in the formulations to enhance and/or prolong product efficacy (Seligy *et al.*, 1997a; Seligy *et al.*, 1997b). Following internalization of the microbe via various routes, virulence factors may be employed to enhance its survival and cause host damage at the cellular level. As a consequence, the host target’s cells may respond by mechanisms that prevent or curtail damage, signalling strategies to solicit aid from other cells, and/or offensive measures to inactivate or eliminate the pathogen.

Figure 1. Potential interactive mechanisms of microbes and animal cells. The major difference between natural bioparticulates which contain specific microbes (A) and commercial microbial formulations (B) is largely one of scale, both in production and release into the environment. During commercial fermentation, the natural organism and its by-products are significantly increased in concentration. As well, several additives may be included to enhance the efficacy of the release and deposit of the commercial product by spraying (C). Following exposure (D), the microbe may produce virulence factors that act to disrupt “normal” cell activity of the target organism (E). At the cellular level, the target organism may respond with offensive measures which include recruiting assistance of many cell types through signalling (F), some with the capacity to cause surface immobilization or engulfment of the microbe (G) and/or produce antimicrobial agents (H; see also Table 1). As well, these cells may alter their metabolism to compensate for homeostatic imbalances (I). In the absence of an adequate or timely response by the target cell, cell death could result leading to loss of tissue and organ function, or even death of the host organism.

which are human pathogens. It is known that the PIB is produced within the sporangium during maturation of the spore. It appears as a crystalline inclusion body, varying in shape according to the *Bt* subspecies that it was obtained from (Beegle and Yamamoto, 1992; Cannon, 1996; Somerville, 1971; see also section 3.1). For example, the PIB of *Bt* subspecies *kurstaki* (*Btk*) is bipyramidal, while that of *Bt* subspecies *israelensis* (*Bti*) is spherical and that of *Bt* subspecies *tenebrionis* is cuboidal. In all cases, it is unclear how these structures are actually formed, and what are their compositions and natural functions. It is known that they are composed largely of ordered aggregates of various δ -endotoxins in precursor form (pro- δ -endotoxin). Their presence and apparent preferential action following activation in the host insect are considered to be the primary reason for order-specific insecticidal activity. The exact composition of PIBs and other proteins produced by *Bt* cells is not known and can vary depending on growth phase and production methods. Aside from the δ -endotoxins, other constituents reported to be part of the PIB are the cytolytic endotoxins (eg. the CytA of *Bti* PIB), proteases (Chilcott *et al.*, 1983a; Nickerson, 1980; Thurley *et al.*, 1985) and nucleic acids (Bietlot *et al.*, 1993; Seligy *et al.*, 1997b). In comparison to the PIB, much less is known about the role of *Bt* spores in the toxification process, but it has been recognized as being important (Asano and Hori, 1995; Estruch *et al.*, 1996; Walther *et al.*, 1986; van Frankenhuyzen, 1994; Yu *et al.*, 1997; Zhang *et al.*, 1995). *Bt* has been shown to be closely related to two human pathogens, *Bacillus anthracis* (*Ba*) and *Bacillus cereus* (*Bc*) by serotyping (flagellar antigen), rRNA sequence similarity, and more critically, chromosome mapping using DNA hybridization and marker probes, and virulence-associated gene detection by polymerase chain reaction (PCR) (Ash *et al.*, 1991; Carlson *et al.*, 1996; Seligy

et al., 1997b). Some researchers have contested that *Bt* is a rightful variant of *Bc* (Carlson and Kolstø, 1993; Gordon, 1977; Zahner *et al.*, 1989). Furthermore, *Bt* cells have similar mammalian cell-like growth and nutrient utilization characteristics, and enterotoxins of *Bc* have been detected in *Bt* culture medium (Beggs *et al.*, 1996; Damgaard, 1995; Seligy *et al.*, 1997a). This close relationship with potential human pathogens has raised serious concerns about the safety of commercial BT products, considering their compositions (see section 1.3) (Dixon, 1994; Drobniowski, 1994; Seligy *et al.*, 1997b; Swadener, 1994).

1.3 Commercial Insecticides Derived from *Bt* Subspecies

Insect pests cause socio-economic and material resource losses, particularly in the forestry and agricultural sectors; they can also be vectors for human diseases. The former are due mainly to larvae of foliage-eating moths and beetles, whereas the latter are the result of blood-sucking dipterans such as mosquitos and blackflies. Recent annual estimates for insect-induced damage range in the billions of dollars (McEvoy, 1996).

The use of chemical insecticides such as organophosphates, pyrethroids and carbamates to control insect pests has evoked concern because of their environmental persistence, related effects on human health, non-target insects, birds and animals, and induced pest resistance (Shand, 1989). These pesticides make little distinction between larval adult stages. In contrast, biological-based agents (“biopesticides” or “bioinsecticides”) used with or even as replacements of chemical insecticides, are viewed as potentially safer, economical alternatives. Their target is usually the feeding larval stage and no other. Since their rather obscure introduction over 40 years ago, the use of bioinsecticides has increased

Mignone, 1995; Beegle and Yamamoto, 1992; Cannon, 1996; Starnes *et al.*, 1993). In contrast, commercial products of *Btk* and *Bti* are poorly described. They are produced by large-scale fermentation (Bryant, 1994). Their compositions have only recently been publicly described. In appearance, they are viscous and very heterogeneous, containing intact and partial PIBs and certain formulation additives to improve the efficacy and possibly the stability or shelf-life of the products, but most importantly, they also contain high levels of viable spores ($\sim 10^{10}$ spores/mL) (Seligy *et al.*, 1997a, 1997b; Tayabali and Seligy, 1995).

The conventional way commercial BT products have been described focuses on the use of the term potency. This term, expressed in international units (I.U.) of activity per quantity of BT product (mg or mL), refers to relative “toxic activity” based on bioassays in which the product is mixed into an insect diet (for lepidopteran larvae) or aqueous medium (for dipteran larvae) and the number of dead larvae is enumerated after a period of four to seven days. The actual I.U. reported on product labels is presumed to correspond to that dose at which 50% mortality occurs (LD_{50}) relative to a *Bt* standard (Beegle and Yamamoto, 1992). It is in fact an ambiguous, non-physical unit because it does not specify the concentration of any particular product constituent as active ingredient. The reference *Bt* standard originally used was an acetone precipitate (powder) of a *Bt* subsp. *thuringiensis* (strain E-61) scale-up culture. The powder was arbitrarily given a potency of 1000 I.U./mg (Burgess, 1966). For products derived from *Btk*, the reference strain has been HD-1-S-1971 (Dulmage *et al.*, 1973). The HD-1 refers to the initials of the isolator, Henri Dulmage, and strain number one, “S” signifies “standard” and “1971” indicates the year it was produced. Compared to E-61, the HD-1-S-1971 gave a potency of 18,000 I.U./mg. After 1980, another

derivative, HD-1-S-1980, was adapted for use (Beegle *et al.*, 1986), which produced 16,000 I.U./mg relative to E-61; it is still being used today for *Btk* standardization. The standard strain available for determining *Bti* potency is IPS-82 with a potency of 15,000 I.U./mg (McLaughlin *et al.*, 1984). Often, BT products may have no description of dosage (I.U./mg or L), but rather only the percentage of “active ingredient” based on total product is given on labels. The I.U. or percent active ingredient is often interpreted incorrectly as being equivalent to the amount of δ -endotoxin present.

1.4 *Bt*-induced Toxification of Insects

The vast majority of studies that demonstrate *Bt* specificity have used protease activated δ -endotoxin purified from late-sporulation cultures of non-commercial strains (Estada and Ferre, 1994; Himeno *et al.*, 1985; Johnson, 1981; Johnson and Davidson, 1984; Masson *et al.*, 1989; Murphy *et al.*, 1976; Vachon *et al.*, 1995; Yoshida *et al.*, 1993). There are no detailed published reports on yields from actual commercial products.

The methods required to either purify precursor, pro- δ -endotoxin or processed, activated δ -endotoxin are very much non-trivial. They are complicated to execute, time-consuming and inefficient in yield. Furthermore, the purity of these preparations is often questionable. However, testing of individual pro- δ -endotoxins from several different *cry* gene products has shaped current thinking of how the pro- δ -endotoxins may act as toxins, even though samples in key work reported by Fast (1977) were later recognized as contaminated with *Bt* spores (van Frankenhuyzen, 1994). The first step in toxification would involve the ingestion by the target larvae of BT product droplets on foliage (*Btk*) or in H₂O (*Bti*). The

PIB which may have up to 75% of its total protein as pro- δ -endotoxin (130 to 137 kDa) (Seligy *et al.*, 1997b), is solubilized by target larvae through oral mastication, alkalinity of the midgut and proteolytic cleavage by either gut cell or PIB-associated proteases (Gill *et al.*, 1992). The resultant "activated" toxin, a truncated version of the pro- δ -endotoxin measuring 62 to 68 kDa for *Btk* and 72 to 78 kDa for *Bti*, is thought to bind to brush border cell receptors (Cannon, 1996). These cell-surface receptors have been tentatively identified from *in vitro* experiments as aminopeptidase N for Cry1Ac and Cry1C, and cadherin-like protein for Cry1Ab (Knight *et al.*, 1994 and 1995; Luo *et al.*, 1996; Sangadala *et al.*, 1994; Vadlamudi *et al.*, 1995). Upon binding to the brush border receptors, the truncated toxins are then thought to insert into the cell membrane, causing the formation of pore-like structures. The disruption of membrane ion permeability by this pore-like structure leads to cell lysis. On the macro-scale, the earliest consistent larval response to BT product exposure is the loss of feeding activity which is followed by eventual death due to starvation and/or bacteraemia. Loss of feeding activity is often measured indirectly by changes in rate of damage to foliage (van Frankenhuyzen, 1990).

In practically all literature reviews, details on larval death are only vaguely described (Aronson *et al.*, 1986; Avignone-Rossa and Mignone, 1995; Cannon, 1996; Gill *et al.*, 1992; Powles and Rogers, 1989) and the role of infection by the spore component of BT products in insect pathogenesis is only emphasized in a few recent studies (Seligy *et al.*, 1997a, 1997b; Tayabali and Seligy, 1995, 1997; van Frankenhuyzen, 1994) and the early work of Ignoffo (1962). A comprehensive review of the literature over the past 30 years shows that the role of the spore has not been investigated in any critical detail, particularly spores in commercial

BT products. However, very recently some toxic substances produced by *Bt* vegetative cells in culture have been reported (Asano and Hori, 1995; Damgaard, 1995; Estruch *et al.*, 1996; Ikezawa *et al.*, 1989; Walther *et al.*, 1986; Zhang *et al.*, 1995).

Another toxin found so far which is peculiar to purified *Bti* PIBs is the 28 kDa cytolytic (CytA) endotoxin which promotes lysis of both insect and mammalian cells *in vitro* (Chilcott and Ellar, 1988; Mayes *et al.*, 1989). It is not known what the natural function of the CytA toxin is. Other than CytA, there are few studies that examine toxicity of other *Bt* byproducts which may be present as part of the formulation or that result from spore germination and proliferation.

Recent evidence (after the initiation of this thesis) suggests that high-level *in vivo* toxicity is dependent on factors that might enhance the activity of the δ -endotoxin (ie. a synergism) or act in a separate mechanism to damage target larvae (Asano and Hori, 1995; Estruch *et al.*, 1996; Hori *et al.*, 1996; Tayabali and Seligy, 1995; Zhang *et al.*, 1993, 1995). As shown in Figure 1, these virulence-like factors would include vegetatively-produced hydrolytic enzymes such as proteases, glucanases, and lipases (Aly, 1985; Beggs *et al.*, 1996; Chiang *et al.*, 1986; Tayabali *et al.*, 1996). For example, the *Bt* exoprotein phosphatidylinositol-specific phospholipase C (PI-PLC) has been shown to release a number of eukaryotic membrane proteins anchored by phosphatidylinositol (PI) (Ikezawa *et al.*, 1983; Taguchi *et al.*, 1980). In doing so, PI-PLC can affect signal transduction within target cells by forming the products diacylglycerol (DAG), which activates protein kinase C, and inositol-1,4,5-triphosphate, which leads to mobilization of intracellular calcium stores (Divecha and Irvine, 1995). PI-PLC is considered a virulence factor of pathogenic bacteria such as *B.*

the β -exotoxin which is not permitted in currently-used commercial formulations because of its lethality to non-target organisms. Most of the studies were apparently often not true field evaluations as they used sterilized soils in artificial microcosm environments. Furthermore, results for the same non-target organism studied by different researchers were often variable and contradictory because there were no attempts to establish standardized assays.

In aquatic environments, the toxicity of *Bti* spores, PIB crystalloids and even commercial *Bti*-BT products (eg. Vectobac 12AS and Teknar HP-D) has been tested using non-target insects, crustaceans, molluscs and fish (Chilcott *et al.*, 1983b; Houston *et al.*, 1989; Merritt *et al.*, 1989; Palmer, 1993; Purcell, 1986; Sinegre *et al.*, 1980; Wipfli *et al.*, 1994). The toxicity observed was against biting, gnat-like midges (Chironomid), mayflies and caddisflies (Palmer, 1993; Sinegre *et al.*, 1980), but dosages were either not stated or not comparable to those used in commercial applications. Tests on mammals, including mice, rabbits, rats and sheep (Hadley *et al.*, 1987; Mayes *et al.*, 1989; Siegel *et al.*, 1987; Thomas and Ellar, 1983) indicated that *Bti* PIB was toxic because of the expression of the CytA toxin (see previous section 1.4) (Mayes *et al.*, 1989; Thomas and Ellar, 1983). Spore-containing samples (4×10^7 spores / 100 μ L) of either *Btk* or *Bti* were not toxic if administered orally over a seven day period, but assessment of immune response effects at later stages was never carried out. Spore-containing samples caused high levels of mortality in as early as 2 h if at least 10^7 spores were injected into the blood (intravenous), peritoneum or cerebrum of mice and rats (Roe *et al.*, 1991; Siegel and Shadduck, 1990; Siegel *et al.*, 1987; Thomas and Ellar, 1983; Tsai *et al.*, 1995). One crucial gap in safety data concerns the collection of rigorous, comparative baseline data on insect versus human cell exposure effects to BT products and

their subcomponents. After conducting an extensive review of the literature on human exposure to *Bt*, it was apparent that some information was available on human health, but very little had involved rigorous proof of actual exposure (internal) or been done under controlled conditions using actual contemporary BT products. The following sections (1.5.1 and 1.5.2) are intended to summarize information from the literature regarding effects of *Bt* on humans and mammalian cell systems.

1.5.1 Effects on Humans

The first *in vivo* study on *Bt* exposure to humans was reported in 1959 by a BT product manufacturer (Bioferm Corporation and Roxney-Hixon Laboratories) to support their registration of the *Btk* product, Thuricide (Fisher and Rosner, 1959). In this study, 18 human volunteers were asked to ingest one-gram capsules of Thuricide daily for five days ($\sim 3 \times 10^9$ spores/day). Five of these subjects also inhaled (both orally and nasally) 100 mg dry powder of Thuricide daily for five days (ie. an additional 3×10^8 spores/day). Although this report lacked experimental details, such as the actual absorbed dose, type of capsules used and if any carriers were included, all findings indicated that no illnesses had occurred after four to five days. Since the 1970's, BT products have increased in potency by 11 to 16X through use of more insect-virulent *Bt* strains (Beegle and Yamamoto, 1992). In a 1977 study of human volunteers exposed to the product Entobakterin containing *Bt* subsp. *galleriae* (Pivovarov *et al.*, 1977), seven of the eight individuals fed either 10^6 to 10^8 *Bt*/g custard or 10^6 to 10^7 *Bt* mixed with 4 g of dessert, exhibited symptoms of food poisoning, which occurred approximately eight hours later. These symptoms included: nausea, eruction, vomiting, stomach cramps, tenesmus and diarrhea. The main conclusion was that *Bt*-based

commercial products can cause symptoms indistinguishable from those observed with *Bc* food poisoning. Since then, there have been several reports of accidents involving *Btk* and *Bti* exposure that resulted in transient infection. A much quoted case is that of an occupational exposure reported by Samples and Buettner (1983) in which an 18-year-old farmer accidentally splashed by *Btk*-based Dipel, developed a corneal ulcer in his right eye. This infection was successfully treated with two antibiotics, gentamicin (40 mg/mL) and cefazolin (50 mg/mL). Bacteria isolated from the ulcer were shown to be gram-positive rods whose spores were associated with PIB-like structures. Furthermore, both the isolate and *Btk* cultured from Dipel were shown to be equally toxic to larvae of Mediterranean flour moth, greater wax moth and southern house mosquito. No diagnostic genetic assays were carried out. In another case reported about the same time, a student in a laboratory accidentally pricked the web-space between his third and fourth fingers with a syringe needle containing a liquid mixture of *Bti* spores and PIBs (Warren *et al.*, 1984). Within hours, the student's finger became discoloured and the entire hand was edemous. Even with intravenous doses of erythromycin, gentamicin and benzylpenicillin (2 to 4 g every 4 h), complete recovery required five days. Cultures of samples taken from the puncture site produced 6×10^8 cfu/mL of *Bti* and another bacteria, *Acinetobacter calcoaceticus* var. *anitratus*, which is normally present on human skin. Culture supernatants (filtrate) from each bacteria grown separately were not lethal when injected into mice (dose not stated), but death occurred when filtrates were obtained from co-cultures. The authors suggested that proteases from either *Bti* or *Acinetobacter* activated the pro- δ -endotoxin, or that germination of *Bti* spores resulted in bacteremia. Further investigations were not done to determine the exact mechanism of the

skin, chapped lips and nose, and throat and eye irritation at about the same time the spray took place. However, actual measurements of acquired dose were not taken. Aside from this, no community “unwellness” could be attributed to the time when the spray operation was conducted. A few years later, a survey conducted by Bender and Peck (1996) following Foray 48B application in Victoria, reported 16 cases of illnesses. Most of these individuals (11) felt that their existing condition (asthma, allergies and environmental/chemical sensitivities) made them more susceptible to the *Btk* sprayed, and 10 of these people characterized their conditions as severe. This study did not provide any exposure information and therefore could not directly link *Btk* with the reported symptoms.

Another publication involving public exposure to *Bt*, concerned an investigation of a gastroenteritis outbreak in an Ottawa chronic care facility (Jackson *et al.*, 1995). In this incident, *Bc* and *Bt* were isolated from spice (onion powder) and human stools. The *Bt* isolates were only distinguishable from *Bc* when microscopical methods were used to identify the PIB. Stools from four of the five patients contained *Bt*, but no *Bc*. These patients showed symptoms of nausea, vomiting and diarrhea, similar to the effects exhibited in *Bc*-related illness. Supernatants from both *Bt* and *Bc* isolates were shown to cause destruction of *in vitro* cultured McCoy cells (a type of mouse fibroblast) at 24 h exposure, but further investigations to identify the cytotoxin were not done. In all of these studies, the actual exposure dose was not reported or determined.

Very recently, Bernstein and our laboratory (Bernstein *et al.*, 1997, submitted for publication) conducted a detailed evaluation of health effects of farm workers exposed to BT products. The study included: case history questionnaires, evidence of dose acquisition in

nasal and mouth lavages, analysis of antibody levels (Immunoglobulins E (IgE) and G (IgG)) following *Btk* exposure and allergic reactions to both BT spores and *Btk* vegetative cell extracts. Ocular irritation and dermatitis symptoms were most prevalent, but significant increases in IgE and IgG levels indicated that allergic reactions could develop with prolonged or repeated exposures. Furthermore, these antibodies were reactive to both *Btk* spores and vegetative cell extracts, implying that at least some spores germinated within the workers. This is the first published study describing the potential implication of immunological effects due to commercial BT products.

1.5.2 Mammalian *in vitro* Models

Studies such as the ones outlined in section 1.5.1 underline the need for more information concerning specific cellular effects that might reveal degree of risk to immunocompromised individuals and acceptable guidelines for human exposure levels to BT products. As mentioned earlier, information concerning human cell exposure to whole products was not available before this thesis. However, some experiments concerning *Bti* PIB effects on mammalian cell lines have been reported. In experiments using canine kidney fibroblasts (MDCK), mouse fibroblasts (L929), primary pig lymphocytes and mouse epithelial carcinoma cells (EC2, EC5 and EC6), purified *Bti* PIBs, if not pre-treated at pH 10 to 10.5 with protease, caused no lysis or morphology changes (Gill and Hornung, 1987; Thomas and Ellar, 1983). However, after protease-treatment, these non-commercial *Bti* PIB preparations were toxic according to the trypan blue assay (see section 1.6.2) with lysis occurring in 30 min with the five cell lines and 60 min with human erythrocytes. The LD₅₀'s were found to range from 10 to 80 µg/mL and one to two µg/mL, respectively (Drobniewski and Ellar,

1989; Gill and Hornung, 1987; Thomas and Ellar, 1983). The morphological changes included cell rounding and swelling, followed by membrane blebbing and cell lysis. In contrast, when activated PIB preparations of *Bt* subsp. *kurstaki* or *aizawai* were used to treat the same cell types, no toxicity, lysis or morphological changes were observed over the three to four h exposures (Niishiitsutsuji-Uwo *et al.*, 1980; Thomas and Ellar, 1983). However, these studies did not determine whether *Btk* or *Bti* spores may have or could have caused damage, and no similar studies have been conducted using actual contemporary BT formulations.

1.6 Potential Assays for Assessing the Effects of BT Product Exposure

None of the cell systems previously used to study *Bt* effects have been tested for the direct effects of BT products. Currently, there are several cell lines used to study bacterial and viral pathogenesis and toxicity of xenobiotic compounds. A good number of these cell lines are actually not that well described. This section will briefly summarize the information on potential cell systems and methodologies that may be useful to study the effects of BT product exposure on animal cells.

1.6.1 *in vitro* Cell Systems

Several cell lines have been derived from lepidopterans whose larvae are targeted by *Btk*-based products. These include: CF1 and CF-124 from larval midgut of the spruce budworm (*Choristoneura fumiferana*), Ld652Y from the gypsy moth (*Lymantria dispar*, tissue source unknown), mbn-2 from the Mediterranean fruit fly (*Drosophila melanogaster*), MG1 from larval midgut and TN-368 from ovaries of the cabbage looper (*Trichoplusia ni*) and *Sf9* and *Sf21* from ovarian tissue of the fall armyworm (*Spodoptera frugiperda*). Of

these, only the *Sf9*, *Sf21* and MG1 cell lines are commercially available and can also be cultured as monolayers. The monolayer property is particularly useful as a tissue mimic and for conducting rapid multistep assays. The *Sf9* cell line has been used extensively to test viruses (eg. *AcNPV*, *TIV*) and purified PIB or δ -endotoxins from *Bt* subsp. *aizawai* and *israelensis* (Brown *et al.*, 1977; Czuba *et al.*, 1994; Jahagirdar and Seligy, 1991; Lee *et al.*, 1993; Monette *et al.*, 1997; Schwartz *et al.*, 1991; Smith *et al.*, 1996; Thomas and Ellar, 1983; Vachon *et al.*, 1995; Villalon *et al.*, 1998; Wang and McCarthy, 1997). The MG1 cell line has not been used before this study in any published viral or *Bt* studies to our knowledge, but it should be useful, since it is derived from larval midgut tissue of the cabbage looper which has in turn been used for almost 25 years as a test standard for BT potency determinations (Dulmage, 1975).

Comparison of responses of insect cell lines to those of non-target human cell lines should provide valuable information about specificity and general effects of BT exposure, if any exist. Besides the human erythrocyte study (Thomas and Ellar, 1983), there are no reports of *Bt* exposure effects on human cells. Human colonic epithelial cells are important models for studying the effects of human pathogenic bacteria and toxins (Cocconier *et al.*, 1992, 1993; Gaillard and Finlay, 1996; Hedges *et al.*, 1992; Jung *et al.*, 1995; Kerneis *et al.*, 1992; Segal *et al.*, 1992; Weikel *et al.*, 1992). The two most important cell lines used over the past 15 years have been HT29 and Caco-2 which have the ability to undergo maturation into at least two cell types found in normal colonic tissue (Lu *et al.*, 1992). Another important model to include in the repertoire would be one for liver because of its role in the clearance of xenobiotics from the bloodstream (Parenti *et al.*, 1992). At present, there are

many liver cell lines, but few exhibit differentiation capacity or responsiveness such as Chang liver cells, which are known to engulf large, non-biological molecules (Sit *et al.*, 1994). Since Chang cells mimic liver-specific macrophages, called Kupffer cells, their response during microbial infection and BT exposure would be important to characterize.

Aside from primary culture studies, there are few cell lines for modelling the responses of various white blood cell types. Two that have been used extensively are the multipotent cell lines, K562 cells that are reported to differentiate into erythrocytes, granulocytes and monocytes, and HL-60 cells that differentiate into cells of the neutrophilic lineage (Gallagher *et al.*, 1979, Lozzio *et al.*, 1981). Unfortunately, both of these cell lines do not grow as monolayers, making it hard to carry out quantitative, multistep treatment assays.

1.6.2 Indicators of Cell Injury

To date, there have been no multiple endpoint studies on any component of BT products. Morphology studies have revealed that larvae exposed to *Bt* spores (Aly, 1985; Chiang *et al.*, 1986; Sutter and Raun, 1967) exhibit midgut epithelial cell pathologies such as vacuole formation, blebbing and microvillar destruction. This midgut damage was then followed by larval paralysis and death. The changes in larval midgut pathology and cytoarchitecture observed during δ -endotoxin or PIB exposures are remarkably similar to those seen during treatment with *Bt* spores at 3,000 to 20,000 cfu per larva (Aly, 1985; Endo and Nishiitsutsuji-Uwo, 1980; Johnson, 1981; Lahkim-Tsrer, 1983; Nyouki *et al.*, 1996; Percy and Fast, 1983; Singh *et al.*, 1986; Spies and Spence, 1985).

Although no information on *in vitro* exposure to whole BT product has apparently been done, experiments by Zhang and colleagues (1995) using vegetative cells of *Bt* subsp.

gelechiae show binding and damage to cultured mbn-2 (*Drosophila melanogaster*) and Sf9 cells. Also, studies involving isolated *Btk* and *Bti* PIB exposures have demonstrated that some of the same features observed *in vivo* are also seen *in vitro* (Himeno *et al.*, 1985; Johnson, 1981; Murphy *et al.*, 1976; Nishiitsutsuji-Uwo and Endo, 1979; Yoshida *et al.*, 1993). Upon exposure to activated δ -endotoxin preparations, cell swelling, membrane vesicle formation and detachment from the substratum occurred. The cells that remained attached showed lysis and loss of cytoplasmic contents.

Other than morphology, methods for examining animal cell responses to *Bt* δ -endotoxin have involved measuring cell membrane integrity and osmoregularity through release and uptake of radioactive compounds ($^{51}\text{Chromium}$, ^3H -Thymidine), vital stains (ethidium homodimer, trypan blue), ions (Ca^{2+} , H^+ , Na^+ , K^+) and cellular levels of ATP (Chilcott *et al.*, 1985; Chilcott and Ellar, 1988; Gill and Hornung, 1987; Himeno *et al.*, 1985; Johnson, 1981; Johnson and Davidson, 1984; Masson *et al.*, 1989; Monette *et al.*, 1997; Schwartz *et al.*, 1991; Smith *et al.*, 1996; Thomas and Ellar, 1983; Vachon *et al.*, 1995; Villalon *et al.*, 1998). However, these methods have not been used to study commercial BT products with the intention of resolving which components of these crude fermentations are the most toxic. An effective comparison between insect and human cells could involve the use of a battery of cell proliferation and toxicity indicators similar to those used to study various mammalian cell systems. This would include: scanning electron microscopy for morphology, bioreduction assessment by tetrazolium dyes, diagnostic immunoprobes for detection of key proteins and carbohydrate moieties (see Table 4), measurement of transcription and translation (protein synthesis) events with radioisotopes and DNA integrity

replacement cells secreted high levels of mucous. In this way, it was thought that the infected larvae were able to curtail *Bt* infection. However, no other studies have tested this theory.

Another possible mechanism that cells may use to control microbial infection is to actually produce antimicrobial agents. With the exception of cultured cells from flesh fly (*Sarcophaga peregrina*; Matsuyama and Natori, 1988), fruit fly (*Drosophila melanogaster*; Samakovlis *et al.*, 1992) and mosquito (*Aedes albopictus*; Hernandez *et al.*, 1994), information on expression of antibacterial agents is limited to *in vivo* experiments with insect haemolymph. Haemolymph cells that participate in the immune-like response of insects have been given various names: prohaemocytes, spherulocytes, plasmatocytes and granulocytes. A list of their putative antimicrobial proteins is given in Table 1. The attacins and cecropins produced by *Hyalophora cecropia* can be degraded by proteases (immune inhibitors A and B) of *Bt* vegetative cells (Dalhammar and Steiner, 1984; Kaaya *et al.*, 1987). However, it remains unclear if insect cells actually express these proteins on exposure to microbial-based insecticides.

Unlike the immune system of insects, the immune system of mammals, particularly humans, is well mapped; it involves both cellular and humoral defences (Costa *et al.*, 1997; Huston, 1997). The cellular responses result from: lymphocytes, polymorphonucleocytes (basophils, eosinophils, and neutrophils), monocytes and macrophages. The polymorphonucleocytes migrate the fastest and are the first to arrive at infection or wound sites. They have roles in cytokine production, phagocytosis and initiating allergic and other humoral responses. Monocytes can migrate in the lymph and blood. On entry into tissues, they increase in size forming tissue-specific macrophages which are capable of engulfing up

Table 1: Insect antimicrobial proteins.

Antimicrobial Group	Function and Structure:	Reference
Lysozyme	<ul style="list-style-type: none"> -relative molecular weight of ~14 kDa -cleaves β-(1,4)-glycosidic bond of peptidoglycan layer of bacterial cell wall -mRNA (cDNA) sequences described for <i>Drosophila melanogaster</i> and genetic homologues reported for other animals such as ruminants and chicken 	Daffre <i>et al.</i> , 1994.
Cecropin(s)	<ul style="list-style-type: none"> -relative molecular weight of 4 kDa -active against gram-negative and gram-positive bacteria -cationic peptides forming two α-helicies that promote membrane "pore" formation -cloned cDNA (mRNA) has homology to magainins and mammalian cecropins -produced <i>in vitro</i> by cell lines 	Anderson <i>et al.</i> , 1990. Bevins and Zasloff, 1990. Hernandez <i>et al.</i> , 1994. Lee <i>et al.</i> , 1989. Moore <i>et al.</i> , 1989. Samakovlis <i>et al.</i> , 1992.
Defensin	<ul style="list-style-type: none"> -relative molecular weight of 4 kDa -forms channels in bacterial cell membranes -not related to mammalian defensin(s) -has 6 cysteines forming 3 disulphide bonds 	Hoffman and Hetru, 1992. Lehrer <i>et al.</i> , 1991.
Attacin Sarcotoxin Diptericin	<ul style="list-style-type: none"> -relative molecular weights of 8-27 kDa -mostly active against gram negative bacteria -no cysteines, mostly cationic -sarcotoxin is produced <i>in vitro</i> by cell lines 	Ando and Natori, 1988. Dimarcq <i>et al.</i> , 1988. Sun <i>et al.</i> , 1991. Matsuyama and Natori, 1988.
Apidaecins Abaecin	<ul style="list-style-type: none"> -relative molecular weights of 2-4 kDa -primarily active against gram-negative bacteria, but also gram-positive bacteria -isolated only from honey bee -cationic and heat-stable 	Casteels <i>et al.</i> , 1989.

to 100 bacteria and then inactivating them with oxygen radicals (nitric oxide, superoxide or hydrogen peroxide) and enzymes (myeloperoxidase, lysozyme). Some examples are the Kupffer cells of the liver, the microglial cells of the central nervous system and alveolar, splenic or peritoneal macrophages (Rappaport, 1980). Engulfment is the primary mechanism used by some immune cells for removing foreign material like bacteria. In some cases, microbes have exploited this pathway to gain entry into phagocytic cells or cross cell barriers (eg. gut epithelium). Bacteria such as *Listeria*, *Salmonella*, *Shigella* and *Yersinia* species are dependent on intracellular entry for completion of their cell-cycle (Finlay and Falkow, 1997). The humoral response provides a sustained immunity against microbes by B-lymphocyte production of antibodies of various classes (eg. immunoglobulin E which is involved in the allergic response and immunoglobulin G which is the major circulating antibody) and are dependent on “antigen-presenting” macrophages and T-lymphocytes which circulate both systemically and close to mucosal epithelia (Williams, 1998). Besides antibodies, another component of humoral defence is the complement system produced by endothelial cells lining blood vessels. This system consists of a number of factors that act coordinately in a cascade of interactions to eliminate microbes (Brock and Madigan, 1988).

Another defence mechanism is the “heat shock response” involving the expression of several classes of proteins. These “heat shock proteins” (hsp) may actually be stimulated by a variety of cellular stresses and are far more ubiquitous than the immune system. Although they are best described for mammals, insect cells have been shown to express hsp’s which are closely related to those of mammals (Baldaia, *et al.*, 1987; Bultmann, 1986; Carvalho and Rebello, 1987; Marin *et al.*, 1994; Ruder *et al.*, 1989; Vazquez *et al.*, 1993; Vincent and

Tanguay, 1979; Whyard *et al.*, 1986). The production of mammalian hsp has been shown to occur during cell exposure to microbes (Kaufmann *et al.*, 1991; Li *et al.*, 1992, Yoshikai, 1992), but the precise role that these proteins play in modulating microbe-exposure is not well understood. For example, human 60 kDa (hsp60) and 70 kDa (hsp70) protein families have been described and implicated in mitochondrial and general intracellular nascent peptide folding, oligomerization and translocation. They may act to prevent denaturation of nascent and existing proteins to ensure cell survival and even differentiation (Beckmann *et al.*, 1990; Craig *et al.*, 1993; Lu and Seligy, 1992; Stuart *et al.*, 1994). One proposed function of vertebrate hsp's is to provide a signal for phagocyte-mediated elimination of bacterial and viral pathogens, and also damaged cells (Li *et al.*, 1992; Yoshikai, 1992). Information on the highly conserved hsp70 family reveals that two of its members, the 72 and 73 kDa proteins are modulated differently (Craig *et al.*, 1993). The 72 kDa protein is thought to be inducible with several different cell stressing stimuli, whereas the 73 kDa protein is thought to be constitutively produced. No information is currently available on the effect of BT products on animal cell hsp's.

The terminal response to target cell damage induced by microbes, is death. During the development of this thesis, information from the literature on cell death has increased exponentially. Since 1990, 18,000 papers have been published on this topic (Garfield and Melino, 1997). Therefore, for practical purposes, only the basic features of the different types of cell death are summarized, based on reviews by Buja *et al.*, 1993; Kroemer *et al.*, 1995 and Steller, 1995. Generally, two types of cell death have been described. One type is referred to as necrosis and the other is "programmed cell death" (including apoptosis). In necrosis,

1.7 Research Objectives

Bioinsecticides derived from commercial fermentations of *Bt* subspecies have been under development for over 40 years. While considerable effort has been made to improve application and efficacy (product formulation), the actual cellular and molecular mechanism(s) involved in the pathogenesis of target organisms (insect pests) and non-targets such as humans are still under investigation. An extensive review of the literature indicates that there are abundant reports on the possible mode of action of the δ -endotoxins based on activated, proteolytically processed and purified forms. However, in contrast, there is little information on the biophysical characterization and toxicology of actual BT products. In particular, it is not clear how these products actually potentiate toxicity in real field application. The primary intent of this thesis was to investigate these broad issues through establishment of standardized, *in vitro* toxicology assays to enable side-by-side comparisons of the responses of insect and human cell lines to current BT products. This has actually never been done before. In the second phase of the work, efforts were directed at determining what component(s) of these products might be the most toxic and responsible for generating target cell changes observed with exposure to whole BT products. In this context, and in inference from empirical data produced here, the central hypothesis of this thesis is that conventional BT products are equally toxic to insect and human cells as a result of ingredients other than the δ -endotoxins (eg. spores and their byproducts). Some of the key sub-objectives and experimental methods are discussed in subsequent sections.

1.7.1 Development of *in vitro* Bioassays for BT Product Toxicology

As mentioned previously, when this thesis was initiated, there were no comparative

selection of two insect cell lines and six human cell lines. For insect cells, *Sf9* is well-known, commercially available and has already been used by this and other laboratories for studies on toxicity of insect viruses and purified PIB-associated proteins like δ -endotoxins (Czuba *et al.*, 1994; Monette *et al.*, 1997; Schwartz *et al.*, 1991; Smith *et al.*, 1996; Thomas and Ellar, 1983; Vachon *et al.*, 1995; Villalon *et al.*, 1998). The MG1 cell line is also commercially available, but much less studied. The MG1 cell line was chosen because of its derivation from larval midgut of the cabbage looper which has been used in *in vivo* testing of BT product potency (Dulmage, 1975). Of the six human cell lines evaluated and partly characterized here (see Table 3, page 35), two systems were especially useful for data collection. One of these, HT29, is a very popular colonic cell system which has been used to study microbial gut cell interactions, including effects of pathogenic bacteria (Cocconier *et al.*, 1992, 1993; Gaillard and Finlay, 1996; Hedges *et al.*, 1992; Jung *et al.*, 1995; Kerneis *et al.*, 1992; Segal *et al.*, 1992; Weikel *et al.*, 1992). However, it has never been used to evaluate the effects of spores of any type, BT products or δ -endotoxins. Another cell line that received attention was the Chang liver cell because of its Kupffer-like phagocyte activity and potential response to bacteria-containing BT products. In addition to comparisons with untreated cells, studies were conducted to investigate the most important exposure-related difference between insect and human cells such as temperature. Temperature of exposure may influence product efficacy against insect pests in natural environments, and the ability of BT to cause injury to non-target organisms. This is particularly relevant since detailed studies on growth kinetics, pH and temperature have shown that the optimal growth conditions for *Btk* and *Bti* are the same as those of human cells (Seligy *et al.*, 1997a).

1.7.3 Toxicity of Commercial BT Product Subcomponent Constituents

There has been considerable effort made to describe the action of the truncated δ -endotoxin, but there is little rigorous study documenting the effects of other BT product components. Recent reports from our laboratory have shown that the commercial BT products contain large numbers of spores ($>10^{10}$ per mL), PIBs (containing both pro- δ -endotoxins and truncated, activated δ -endotoxins), nucleic acids and possibly other toxins such as proteases and lipases, antibiotic hydrolases, formulation additives and fermentation by-products (Beggs *et al.*, 1996; Rancourt *et al.*, 1996; Seligy *et al.*, 1997a; Tayabali *et al.*, 1996). In this thesis, five BT products were evaluated for toxicity, along with individual components. As additional controls, studies were conducted on bacteria that are commonly used in biotechnology research and laboratory investigations (eg. *Bacillus subtilis*, *Escherichia coli*). Since *Bt* spores have been observed to grow in insect cell and human cell culture media, part of the investigation was directed at using an antibiotic to inhibit spore germination and vegetative cell growth in order to assess the effects of BT product additives and PIBs. Also, several virulence-enhancing byproducts that may result from *Bt* proliferation were tested. Some of these components are actually commercially available from related *Bt* subspecies (eg. phosphatidylinositol-specific phospholipase C) or *Bc* (eg. diarrhoeal enterotoxin). In later investigations, published methods for purification of PIB-associated proteins and newly reported Vegetative Insecticidal Protein (VIP) were used to obtain them from BT products and vegetative cultures in order to conduct a side-by-side assessment of heterogeneity and what may be the most cytotoxic agent(s) generated in BT products.

Table 2. General Description of Commercial BT Products

Commercial Product (lot #)	Supplier	Product Type	Subspecies and Strain ^b	I.U./mL x 10 ⁶	spores/mL x 10 ⁹ (spores/I.U.) ^b	spore:PIB ratio ^c
DiPel*WP ^a (5188(13))	Abbott Quebec	Powder	<i>kurstaki</i> , HD-1	4.2	5.9 ± 1.3 (~1400)	11:1
Foray 48B (BBN 6306)	Novo/Abbott Quebec	Liquid	<i>kurstaki</i> , HD-1	12.7	31.5 ± 7.0 (~2480)	2:1
Foray 76B (BBN 7019)	Novo/Abbott Quebec	Liquid	<i>kurstaki</i> , HD-1	20	39.8 ± 8.7 (~2460)	2:1
Thuricide (92023353, 92023538)	C.I.L. Quebec	Liquid	<i>kurstaki</i> , HD-1	4.2	10.0 ± 2.1 (~2380)	4:1
VectoBac 12AS (08-306-N9)	Abbott Quebec	Liquid	<i>israelensis</i> , H-14	1.3 ^c	29.4 ± 7.6 (~22 260) ^d	3:1

^a Referred to as Dipel in this thesis. Since this product was a powder, a working stock equivalent to Thuricide I.U. content was prepared (2.625 g Dipel / 10 mL ddH₂O).

^b Data from Seligy *et al.*, 1997a.

^c This value was experimentally determined from product label information (1200 I.U./mg).

^d The I.U. of *Bti* -based product is not comparable with that of *Btk* products, since they are calibrated by the manufacturer using different insect orders (dipteran versus lepidopteran). For exposure experiments, the cfu content of Vectobac was used instead of I.U.

^e These values were determined by enumeration from SEM micrographs.

Table 3. Description of Cell Lines and Some Experimentally Derived Properties.

Cell Line	Animal and Tissue Source (Passage Number)	Supplier (Lot Number)	Propagation Medium	Temperature (Doubling Time)*	Cell Detachment (Subcultivation Ratio)*
Caco-2	<i>Homo sapiens</i> colon adenocarcinoma (17)	ATTC, Rockville, MD (F-11225)	Dulbecco's Modified Eagles Medium + 25 mM Glucose + 10 % (v/v) FBS	37°C (28 ± 2 hrs)	0.25% (v/v) Trypsin (1:4)
Chang	<i>Homo sapiens</i> liver (258)	ATTC, Rockville, MD (F-11873)	Dulbecco's Modified Eagles Medium + 25 mM Glucose + 10 % (v/v) FBS	37°C (16 ± 2 hrs)	0.25% (v/v) Trypsin (1:8)
Hep-G2	<i>Homo sapiens</i> hepatoblastoma (75)	ATTC, Rockville, MD (F-11225)	Dulbecco's Modified Eagles Medium + 25 mM Glucose + 10 % (v/v) FBS	37°C (23 ± 2 hrs)	0.25% (v/v) Trypsin (1:4)
HL-60	<i>Homo sapiens</i> promyelocytic leukaemia (21)	ATTC, Rockville, MD (F-11917)	Dulbecco's Modified Eagles Medium + 25 mM Glucose + 10 % (v/v) FBS	37°C (24 ± 2 hrs)	suspension culture (1:4)
HT29	<i>Homo sapiens</i> colon adenocarcinoma (129)	ATTC, Rockville, MD (F-12101)	Dulbecco's Modified Eagles Medium + 25 mM Glucose + 10 % (v/v) FBS	37°C (22 ± 2 hrs)	0.25% (v/v) Trypsin (1:4)
K-562	<i>Homo sapiens</i> chronic myelogenous leukaemia (unknown)	ATTC, Rockville, MD (F-11533)	Dulbecco's Modified Eagles Medium + 25 mM Glucose + 10 % (v/v) FBS	37°C (18 ± 2 hrs)	suspension culture (1:8)
MG1	<i>Trichoplusia ni</i> cabbage looper midgut of fifth instar larva (unknown)	Invitrogen, San Diego, CA (120721)	Grace's Insect Medium 3.32 g/L lactalbumin hydrolysate, 3.32 g/L yeastolate, 0.6 g/L glutamine, 10% FBS	27°C (31 ± 2 hrs)	0.25% (v/v) Trypsin (1:4)
Sf9	<i>Spodoptera frugiperda</i> fall armyworm ovary (3)	Invitrogen, San Diego, CA (121117)	Grace's Insect Medium 3.32 g/L lactalbumin hydrolysate, 3.32 g/L yeastolate, 0.6 g/L glutamine, 10% FBS	27°C (30 ± 2 hrs)	sterile cell scraper (1:4)

*Determinations made during thesis work

Canada), and uranyl acetate (BDH).

For bioreduction activity assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; lot 102H50195), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT; lot 36HO710), dimethylsulphoxide (DMSO), and phenazine methosulphate (PMS) were purchased from Sigma. The MTT was made up as a stock solution of 10 mg/mL in PBS and stored in the dark at 4°C until use. XTT solutions were made just prior to use in Grace's medium with 10% (v/v) FBS. Bioreduction assay inhibitors, sodium azide and 2,4-dinitrophenol were from Sigma. The calcium ionophore, A23187, was purchased from Boehringer Mannheim (PQ, Canada).

For polypeptide analysis, acrylamide and bisacrylamide (N',N'-bis-methyleneacrylamide), glycerol, prestained molecular weight markers (containing: lysozyme, β -lactoglobulin, α -chymotrypsinogen, ovalbumin, bovine serum albumin, phosphorylase B and myosin heavy chain) and Tris were purchased from GIBCO/BRL. Ammonium persulfate (APS), bromophenol blue, Coomassie blue R-250, dithiothreitol (DTT), glycine, 2- β -mercaptoethanol, silver nitrate sodium dodecyl sulfate (SDS) and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Sigma. Proteins were blotted onto polyvinylidene difluoride (PVDF; Bio-Rad) membranes. Blocking solution consisted of powdered milk (Nestle, ON, Canada). Immunoprobes that were tested are described in Table 4. Bovine serum albumin (BSA; lot 129F00506), 4-chloro-1-naphthol (lot 53H3458), streptavidin-peroxidase (lots 014H4862, 014H4864, 114H4817, 114H4842) and Kodak X-OMAT AR autoradiographic films were purchased from Sigma.

Purified, natural *Bacillus thuringiensis* phosphatidylinositol-phospholipase C (PI-

Table 4: Immunoprobosc (Antibodies) Screened for Reactivity to Insect and Human Cell Line Proteins

Antibodies (supplier; lot)	Function of Antigen	Expected MW (kDa)	Observed MW (kDa)	Human Cell Reactivity (Titre)	Insect Cell Reactivity (Titre)
Actin (Boehringer-Mannheim; 14030720-04, 14030721-04, 14279421-04)	-major cytoskeletal component; monomer of microfilaments	42	42	Yes (1:100; 10µg/mL)	Yes (1:100; 10µg/mL)
Catalase (Serotec, Oxford, England; 583701)	-antioxidant; detoxification of hydrogen peroxide	240	none	No	No
Cyclin (Boehringer Mannheim; 12906222-04, 12906225-04)	-accessory protein to DNA polymerase; proliferating cell nuclear antigen (PCNA); cell cycle progression is blocked in its absence or inactivation	36	36	Yes (1:40; 5µg/mL)	No
Glutathione-S-Transferase (Cedarlane, Hornby, Ontario; AX3611E)	-catalyst involved in glutathione-mediated detoxification of electrophilic substrates	20	none	Yes (1:20; not given)	No
Granulocyte Macrophage-Colony Stimulating Factor (Cedarlane, Hornby, ON; 401024)	-cytokine; promotes survival and growth of granulocytes and macrophages	18-22	none	No	No
Heat shock protein 25 (Sigma; 072H-4806)	-inhibits microfilament polymerization	25	none	No	No
Heat shock protein 60 (Monosan, Am Uden, Netherlands; 1)	-heat shock protein 60kDa; chaperonin	60	60	Yes (1:10; 10µg/mL)	No
Heat shock protein 70 (Sigma; 102H-4809, 026H4826)	-heat shock protein 70kDa; chaperonin, protein stability/folding; constitutive and inducible forms	72, 73	72, 73	Yes (1:1000; 11µg/mL)	Yes (1:1000; 11µg/mL)
Interleukin-8 (Cedarlane, Hornby, ON; 506075)	cytokine; induces neutrophil migration and activation	8	none	No	No
p53 (Boehringer Mannheim 12986821-01)	-tumour suppressor; binds cdk to prevent cell division	53	none	No	No
P-glycoprotein (Boehringer Mannheim; 12806921-01)	-involved in toxin resistance	170-180	none	No	No
Cu/Zn Superoxide Dismutase (Serotec, Oxford, England; X4367)	-antioxidant; partial detoxification of superoxide anion	30	none	No	No
Tubulin (Boehringer-Mannheim; 11511425-01)	-component of cytoskeleton; monomer of microtubules	50	50	Yes (1:10; 2.0 µg/mL)	Yes (1:10; 2.0 µg/mL)
Tumour Necrosis Factor α (Cedarlane, Hornby, ON; 506051)	-cytokine; regulation of inflammation and host defence	17	none	No	No
Mouse (Sigma; 041H-4862), Rabbit (Sigma; 034H8945) IgG (biotinylated)	-used as secondary antibodies	-	none	(1:2500)	(1:2500)

PLC) was purchased from ICN (lots 58082, 58083). Recombinant *Bacillus thuringiensis* PI-PLC was purchased from Oxford Glycosystems (lot A615202). *Bacillus cereus* enterotoxin standards were part of detection kits from Oxoid (lot 61607) and Tecra (lot 1400623).

For nucleic acid analyses, proteinase K and electrophoresis grade agarose were purchased from GIBCO/BRL. Nucleic acids were transferred to nylon membranes (Zeta-probe GT; Bio-Rad). Micrococcal nuclease (lot 5040584011) for creating nucleosome oligomers was purchased from Pharmacia Biotechnology.

³⁵S-labelled methionine (ICN lot 182-386H1, 1163 Ci/mmol and lot 182-410X3, 553 Ci/mmol), α -³²P-labelled nicotinamide adenine dinucleotide (³²P-NAD; lot J7END1, 550 Ci/mmol and lot S7RND4, 350 Ci/mmol) and gamma-³²P-labelled adenosine triphosphate (³²P-ATP; lot S7RAGX4, 4500 Ci/mmol) were purchased from ICN.

Reagents for buffer solutions were purchased from BDH, Fisher and Sigma and made up as follows: SDS-PAGE running buffer (24.8 mM Tris, 191.8 mM glycine, 3.5 mM SDS, pH 8.3); 2X Laemmli sample buffer (LSB; 125 mM Tris-HCl pH 6.8, 20 % (v/v) glycerol, four % (w/v) SDS, 10 % (v/v) 2- β -mercaptoethanol, 0.01 % (w/v) bromophenol blue); phosphate-buffered saline (PBS: 1.5 mM KH₂PO₄, 16 mM Na₂PO₄, 137 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, pH 6.8); Tris-buffered saline (TBS; 24.8 mM Tris, 137 mM NaCl, 2.68 mM KCl, pH 7.4); Tris-EDTA buffer (TE: 10 mM Tris pH 7.5, 1 mM EDTA).

All reagents and materials were stored according to manufacturers' recommendations for temperature and expiry date.

Methods

The general scheme for the methods used in this thesis is shown in Figure 2. The commercial BT products that were used in exposure experiments are those described in Table 2. Animal cell systems are those described in Table 3. The battery of *in vitro* assays developed and used for animal cell exposure to BT products were also used on BT product subcomponents to identify principle cytotoxic component(s) by partial purification and comparison to known virulence factors.

2.1 Analysis of Commercial BT Products

2.1.1 Dosage of BT Products

The commercial BT products used here (Table 2) are highly viscous, particulate suspensions that require extensive mixing before dispensing into aliquots. For dose-response studies, appropriate dilutions were required to simulate possible delivered dose from field application (Table 5). This could be done by directly aliquoting from the products or dilution of the products. Each dose per well was made up to 10 μ L with isotonic PBS before mixing in the well. Controls (zero I.U.) consisted of 10 μ L PBS alone. The dosing conditions used for BT product exposure experiments are described in Table 5.

2.1.2 Partitioning of BT Products into Subfractions

BT products were separated into non-particulate (liquid) and particulate fractions by centrifugation at 12 000 x G for 10 min. The resulting non-particulate supernatants were filtered (0.45 μ m pore size) to remove any residual spores. Usually a 0.1X stock was made up first and then used to make $\log_{10}$ serial dilutions in PBS. If needed, filtrates were

Table 5. Summary of Parameters Established and Used in BT Product Exposures

CONTAINER	RADIUS (mm)	SURFACE AREA (mm ²)	MEDIUM VOLUME (μL)	^a LIQUID HEIGHT (mm)	SEEDING DENSITY (CELLS/WELL)	HIGHEST BT DOSE (TU/WELL)	AMOUNT OF LSB (μL)
T-80	-	7654	23200	3	8.1×10^6	23200	4600
T-25	-	2372	7200	3	2.5×10^6	7190	1500
6-WELL	17.25	935	2800	3	10^6	2830	500
12-WELL	11.75	434	1300	3	4.7×10^5	1320	250
24-WELL	7.5	177	540	3	1.9×10^5	540	100
48-WELL	5	79	240	3	8.4×10^4	240	50
96-WELL	3.25	33	100	3	3.5×10^4	100	20

^aThese are calculated values which do not include the effect of the meniscus.

concentrated 10 to 20-fold by reverse osmosis, by placing five mL of filtrate into a dialysis tube sealed at both ends, and covering with Ficoll 400 for two to three h at 4°C. The particulate-containing pellet fraction was washed with ddH₂O (or PBS in some cases), before resuspending and adjusting (with PBS) to the original volume of the BT product used.

2.1.3 Preparation of Parasporal Inclusion Bodies from BT Products

Purification of parasporal inclusion bodies (PIBs) from two representative types of commercial BT products (Foray 48B and Vectobac) was done by the method of Thomas and Ellar (1983) to isolate δ -endotoxins from *Btk* and *Bti* cultures. All dilutions, rinses and resuspensions for *Btk* product (Foray 48B) were done with ddH₂O and *Bti* products with 50 mM Tris-Cl (pH 7.5). A 500 μ L aliquot of each product was diluted two-fold and large particulates were disaggregated with ~50 μ L sterile crushed glass and vortexing vigorously for five min. After settling out the glass for 10 min, the tube contents, excluding the glass, were removed and centrifuged at 12,000 x G for 10 min. The pellets were resuspended (500 μ L final) and layered onto a discontinuous gradient consisting of one mL layers of 67%, 72% and 79% (w/v) sucrose for *Bti* PIBs, and layers of 45%, 67% and 87% sucrose for *Btk* PIBs. The polyallomer tubes (13 x 51 mm) containing the step gradients were spun at 26,000 rpm (80,000 x G) using a Beckman SW50.1 rotor for 14 h at 4°C. Following centrifugation, each brownish, opaque “band”, was collected and transferred into clean, sterile 1.5 mL microfuge tubes. The crystal-enriched band, identified by scanning electron microscopy (SEM; section 2.3.2) and protein electrophoresis (section 2.5.1), was diluted with 0.5 mL ice-cold ddH₂O and then centrifuged at 12,000 x G for 10 min to remove residual sucrose. This procedure was repeated three times. A portion of the contents of the PIB preparation was solubilized

in 20 mM Tris, pH 8.0 to give a protein concentration of either 500 or 1000 -fold of the original unconcentrated filtrate. Activity of the concentrated, resuspended pellets were determined experimentally using various bioassays (section 2.4.1).

CFs were subjected to various treatments prior to animal cell exposure. Microtubes containing one mL of CF were incubated at various temperatures (0, 4, 23, 27, 37, 50, 60, 70, 80, 90 or 100°C) and intervals (0.17, 0.5, 1, 3, 5, 120, 720, 1440, 2880 min). CF samples were also subjected to either cycles of freezing (-80°C) and thawing (37°C), EDTA at 0.1, 0.5, 1, 5, 10 mM, one mM phenylmethylsulfonyl fluoride (PMSF), 0.25% trypsin or 100 µg/mL proteinase K. Control samples consisted of Grace's medium treated or supplemented in the same manner as the CF.

To determine whether CF proteins can be phosphorylated *in vitro*, 24-h CF's (1000X) were incubated in the presence of either ³²P-labelled nicotinamide adenine dinucleotide (NAD) or γ-³²P-labelled adenosine triphosphate (ATP), according to the method of Just and colleagues (1992). For this reaction, CF samples were thawed on ice. During thawing, a master mix was made: 10 µCi ³²P-NAD or ³²P-ATP, 50 µL 1 M Tris-Cl (pH 8.0), and 240 µL ddH₂O. The master mix (35 µL) was then aliquoted into 10 sterile microtubes. To these tubes, 15 µL of CF was added (time=0). At 0, 20, 40 and 80 min, 50 µL of LSB (see section 2.5.1) was added to each tube, which was immediately heated for five min at 95°C to denature proteins and stop any enzymatic reaction present. Samples (20 µL) were subjected to SDS-PAGE. Gels were recovered, vacuum-dried and exposed to autoradiograph film for varying lengths of time to detect radiolabelled protein bands. See section 2.5.1 for details on SDS-PAGE gels.

2.1.5 Column Chromatography of *Bt* Culture Filtrates

To enrich for the cytotoxic ingredient from vegetative cell cultures filtrates, several chromatographic techniques were used. Size exclusion chromatography was done using 0.7 x 8 cm columns loaded with beads of Agarose-1.5m (exclusion limit: 1.5×10^6 Da), Sephadex G-100 (exclusion limit: 100 kDa) or Sephadex G-75 (exclusion limit: 80 kDa). Each type of bead was prepared by washing in either 10-20 mM Tris, pH 8.0 or 10 mM triethanolamine, pH 8.0. After equilibrating the columns with several washes of buffer, 100 to 200 μ L of concentrated CF (1000X) was carefully layered onto the top of each column and permitted to absorb. Elution involved passing four column-volumes of buffer through the column and collecting each of the 150 μ L fractions separately in 96-well plates. Anion exchange chromatography was done using a 3 x 0.7 cm column packed with Sephadex DEAE-50 equilibrated in 20 mM Tris pH 7.4. To this column, was loaded 150 μ L of 1000X CF samples. After addition of three column volumes of loading buffer (~750 μ L), 150 μ L of Tris buffer containing 0.2 mM NaCl was added, followed by successive volumes containing increasing amounts of NaCl in buffer (increments of 0.2 mM) to yield a semi-discontinuous elution gradient ranging from 0.2 to 1.0 M NaCl. Ultraviolet-transparent 96-well plates were used to collect fractions so that the approximate protein concentration of each fraction could be determined by extent of light absorption at 280 nm using the Spectramax 250 scanning microwell reader. Each fraction (30-40 in total) from each column was then analyzed by SDS-PAGE and silver staining (section 2.5.1), and also by bioassays to measure changes in morphology (section 2.3.1) and bio-reduction (section 2.4.1) following exposure of insect or human cells to 10 μ L of each fraction. All fractions were stored at -80°C until use.

2.2 Animal Cell Manipulation and Propagation

2.2.1 Insect Cell Lines

As mentioned in section 1.5.1 and described in Table 3, two lepidopteran cell lines were used in experiments for studying exposure to BT products. Both cell lines were maintained at 27°C as suggested by the supplier using medium described in Table 3. The cells were subcultured every two or three days. MG1 cells were detached from the flask surfaces by aspirating off the medium, briefly rinsing the cell monolayer with isotonic PBS and 10 mM EDTA, and then incubating the cells for five min with 0.25 % (w/v) trypsin in Hank's buffered saline. Sf9 cells were physically detached with sterile cell scrapers. For general culture maintenance, the cells were replated at a density of 3×10^5 cells/mL in either T25 (5 mL) or T80 (10 mL) culture flasks.

2.2.2 Human Cell Lines

The human cell lines used for BT exposure were initially subcultured in media recommended by the supplier. To facilitate comparative analyses between cell lines, the human cells were gradually adapted to and subsequently maintained in DMEM with 25 mM glucose, 2 mM glutamine, 10 % (v/v) FBS and 50 µg/mL gentamicin. Like insect cells, the human cell lines were generally maintained and subcultured in either T25 or T80 tissue culture flasks. The HT-29, Caco-2, and Hep-G2 cells were subcultured by trypsinization as described for MG1 cells and were resuspended to 3×10^5 cells/mL in fresh DMEM. Chang cells were resuspended to 1.5×10^5 cells/mL. HL-60 and K562 cells grow as free cells in suspension culture. They were subcultured by diluting the cell cultures in four and eight volumes, respectively, into fresh DMEM.

2.3 Morphological Studies

2.3.1 Phase Contrast and Light Microscopy

Light microscopes of three types were used: an Olympus IMT-2 microscope with Hoffman Modulation Contrast Optics, a Nikon Diaphot microscope equipped with phase contrast optics and a Zeiss Light Microscope. Photographs were taken with either an Olympus OM-4T or Nikon FE2 camera using Scotch 800/3200 ASA colour slide film. Constituents of BT products and six h growth cultures were tested for their ability to react with a commercially available "gram stain" reagent kit. Serial $\log_{10}$ dilutions were made of each *Bt* sample and 5 to 10 μL was smeared onto clean glass slides. Following air-drying and ~10 sec exposure of the underside of the slide to a thermally regulated block set at 100°C, the slides were flooded with crystal violet (one mg/mL) for 30 sec, rinsed with water (30 sec) and then incubated with iodine solution (3.3 g/L iodine, 6.7 g/L potassium iodide, 10 g/L stabilizer) for 30 sec. Following another rinse with water, slides were rinsed with decolourizer solution (50% (v/v) acetone, 50% (v/v) 2-propanol) until no further stain was removed. Slides were then rinsed with water, counter-stained with Safranin solution (6 g/L safranin and 20% (v/v) ethanol), rinsed again with tap water and air-dried before viewing under oil immersion with a 100X objective lens.

2.3.2 Scanning Electron Microscopy (SEM)

Monolayers for SEM examination were grown on fragments of glass coverslips of ~five mm² in size. Cells were fixed with 4 % (v/v) glutaraldehyde in 100 mM sodium cacodylate (pH 7.2), rinsed 2X 10 min with 100 mM sodium cacodylate buffer, post-fixed with 1 % (w/v) osmium tetroxide in 100 mM sodium cacodylate, rinsed 2X 10 min with

ddH₂O and dehydrated with an ethanol wash series of 30%, 50%, 70%, 90% and 100% (for five min each). Following two changes in absolute ethanol, cell-coverslips were placed in porous specimen holders immersed in 100 % ethanol. Samples were dried in a carbon dioxide critical point drier (Autosamdri 814, Tousimis) operating on the automatic setting. Samples were then removed from the specimen holders and mounted sample-side-up on double-sided carbon tape for sputter-coating with 10 nm of gold particles. Samples were viewed with a JOEL JSM6400 SEM operating at 10 kV. BT products at the dilutions used for animal cell exposures were also examined by SEM. BT product was diluted in ddH₂O, dropped onto 0.5 cm² aluminum foil pieces and air-dried. The foil pieces were placed onto double-sided carbon tape. Samples were coated with gold and examined as described for animal cells, except that the microscope was operated at three kV.

2.3.3 Transmission Electron Microscopy (TEM)

To test for preprocessing changes in cell morphology, the cell monolayers were grown on sterile 30 mm² square or 22 mm diameter flexible support films and a piece of each film was placed into wells of a 6-well (3.5 cm diameter) culture dish. Following culture and exposures outlined in Table 5 and section 2.2, cells that had detached from the film surface were recovered as cell pellets, but cells that had remained attached to the film surface were processed for electron microscopy either as monolayers or trypsin-released cells (section 2.2.1). Cell-bound films were briefly rinsed in PBS and fixed for one h at room temperature in 66.7 mM sodium cacodylate buffer (pH 7.2) supplemented with 4 % (v/v) glutaraldehyde and 0.5 mg/mL ruthenium red, an electron-dense stain for acid mucopolysaccharides (Hayat, 1989). The monolayers were then rinsed two times for 15 min each in 100 mM sodium

bioreduction activity assay of Mosmann (1983) was adapted to measure changes in electron transport activity (Tayabali and Seligy, 1995). Cells were seeded into each well of 96-well (microtitre) plates at densities and medium volumes described in Table 5. Medium was either DMEM with 25 mM glucose or Grace's Medium plus supplements, $\pm 10\%$ FBS, 50 $\mu\text{g/mL}$ gentamicin. Following overnight incubation at 27°C or 37°C, the media were removed by aspiration and replaced with 90 μL of fresh media. Gentamicin was included at 50 $\mu\text{g/mL}$ in some wells to test the effects of BT components other than spores. Dilutions of BT were added to culture medium at various points 24 h before the completion of the assay at which time, 11 μL of MTT stock (10 mg/mL) was added and incubated for two h at either 27°C or 37°C. The supernatants were then removed by aspiration and wells were rinsed briefly twice with 100 μL PBS/well to eliminate any optically interfering material. Dimethyl sulfoxide (DMSO; 100 μL /well) was added to each well and microtitre plates were gently shaken for at least five min prior to reading of optical densities at 490 nm using a Molecular Devices Thermomax or SpectraMax250 Microplate Reader. Data were collected with the instrument software (SoftmaxPro), and analyzed using Microsoft Excel (version 5.0) and SlideWrite Plus software. Scanning spectrophotometry (either Spectramax 250 or Beckman DU70) was used to evaluate optimal wavelength for detection of the MTT formazan produced by various cell lines. A potent inhibitor and an uncoupler of mitochondrial activity, sodium azide and 2,4-dinitrophenol respectively, were used as positive controls. The calcium ionophore, A23187, was exposed to MG1 for up to two h at concentrations of 0.5 to 50 μM .

2.4.2 XTT Indicator Assays

The suitability of the tetrazolium dye (2,3-bis (2-methoxy-4-nitro-5sulfophenyl)-2H-

tetrazolium-5-carboxanilide sodium salt (XTT)) which produces a water-soluble formazan product was also examined for use in insect and human cell assays. To determine optimal concentrations of substrate (XTT) and an electron coupling potentiator (phenazine methosulfate; PMS), animal cells and/or BT were incubated with varying concentrations of XTT (0, 370, 750, 1110 or 1500 μM) and PMS (0, 10, 25, 50, 70 μM). Optical density measurements for XTT formazan were taken at 480 nm. For kinetic or "real time" assays, measurements were taken every 15 min for an 18 h period using special feature of Spectromax 250. Only data below the maximum detection limit of spectrophotometer (~ 3.2 O.D.) were used.

2.5 Protein Analyses

2.5.1 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Polypeptides were examined by SDS-PAGE (Laemmli, 1970) using a BioRad Mini-PROTEAN II gel apparatus with a 12 % separating gel (375 mM Tris-HCl (pH 8.8), 0.1 % (w/v) SDS, 12 % (w/v) acrylamide/bisacrylamide (from a stock solution of 29.2 % (w/v) acrylamide and 0.8 % (w/v) bisacrylamide), 0.05 % (w/v) APS and 0.125 % (v/v) TEMED and a four % (w/v) stacking gel (125 mM Tris-HCl (pH 6.8), 0.1 % (w/v) SDS, four % acrylamide/bisacrylamide from the stock solution, 0.05 % (w/v) APS and 0.14 % (v/v) TEMED). Samples were diluted 1:1 with 2X LSB, heated for five min at 95°C and then cooled to RT prior to loading. Gels were run at 120 V for 1.5 to 2 h in electrophoresis running buffer. Staining was done overnight at RT with 0.1 % (w/v) Coomassie R-250 in destaining solution (40% (v/v) methanol, 10 % (v/v) glacial acetic acid), and destaining was

done in the same methanol-acetic acid solution 6 to 8 h at RT.

Alternatively, polypeptides were stained using a non-ammoniacal silver staining protocol (Morrissey, 1981). Gels were first emersed in a glass dish containing 50 mL of fixing solution (10% acetic acid and 40% methanol in ddH₂O) and gently agitated for 30 min at RT, then soaked in a 50 mL solution of 7 % acetic acid and 5 % methanol (in ddH₂O) for 30 min at RT. After this treatment, gels were exposed to 50 mL of 10 % glutaraldehyde for 10 min at RT and then washed with several changes of ddH₂O over a 2-h period. Gels were then soaked in 100 mL of five µg/mL DTT for 30 min and then transferred to 100 mL of 0.1% (w/v) silver nitrate. The silver nitrate exposure was 30 min and then the gels were quickly washed, once with ddH₂O and twice with carbonate developing solution (0.0185% formaldehyde and 3 % sodium carbonate). The gels were then soaked in 100 mL developing solution with gentle agitation until the desired level of staining intensity was obtained. Silver deposition was stopped by adding five mL of 2.3M citric acid for 10 min at RT. Both Coomassie and silver stained gels were stored by soaking them in a solution of 3 % glycerol, 20% methanol (in ddH₂O) for 30 min and drying them on a pre-cut sheet of Whatman 3MM filter paper using a gel dryer (Fisher model SGD-4050) for 2 to 8 h at 80°C.

2.5.2 Immunodetection Assays

Electrophoretic transfer of polypeptides was done according to Towbin and colleagues (1979), but PVDF membranes were used instead of nitrocellulose. Gels were removed from the PAGE apparatus and equilibrated for 15 min at RT in transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol, pH 8.3). The PVDF membranes were pre-wetted (10 sec) in methanol and then in transfer buffer (15 min at RT), along with fibre pads

and Whatman 3MM filter sheets. The transfer cassette was assembled in the following order from cathode to anode: fibre pad, Whatman 3MM, the PAGE gel (with prestained marker on the right), PVDF membrane, Whatman 3MM, fibre pad). The transfer cassette was closed and inserted into the cassette holder. A small stir bar (2 cm) and transfer buffer were put into the buffer chamber and the entire transfer apparatus was placed onto a magnetic stirrer. Transfer was done either overnight at 30 V, RT or for one h at 100 V, 4°C.

Following transfer, PVDF membranes were removed and air-dried on clean Whatman #1 sheets. Prior to beginning the immunodetection protocol, the membranes were re-wetted (10 sec) with methanol and then with TBS for 15 min. Membranes were treated for two h with 5 % (w/v) skim milk in TBS and exposed for one h at RT with one of the probes listed in Table 4. Membranes were drained of excess probe, incubated for one h with the appropriate biotinylated secondary antibody (1:1000; see Table 4) and then one h at RT with streptavidin peroxidase solution diluted with TBS (1:300). The peroxidase reaction was visualized using 4-chloro-1-naphthol as substrate (three mg/mL 4-chloro-1-naphthol in four mL methanol mixed with 0.018 % (v/v) hydrogen peroxide in 20 mL TBS). All antibody dilutions were made in TBS with 3 % (w/v) BSA. Between every step, blots were rinsed with TBS three times for 10 min each at RT.

2.5.3 *in vivo* Radiolabelling of *Bt* and Animal Cells

The production of *de novo* synthesized polypeptides from *Bt*, insect and human cells was assessed by incorporation of a radioactively labelled amino acid (³⁵S-methionine). Animal cells were either pre-labelled, or labelled during the exposure regime. Cells were seeded into 48-well plates at the density shown in Table 5 with media containing FBS and methionine.

After ~18 h for cell attachment, media was removed from the wells and replaced with 100 μ L fresh media, containing FBS, methionine, and 400 μ Ci/mL of 35 S-methionine (final = 8.5 μ Ci/ μ L of medium). After 30 min at either 27°C (insect cells) or 37°C (human cells) cells received 150 μ L of 8-h CF at 0, 1, 5, 10, 50 or 100 (undiluted) % in media without FBS. Following a further incubation of either 5, 10, 20, 30, 60, 120 or 240 min, supernatants along with any detached cells and cellular debris were transferred to microtubes and either frozen at -80°C or centrifuged at 1000 x G for 10 min. The cell pellet (if any) was solubilized with 50 μ L LSB. The cell monolayers were solubilized in 50 μ L LSB and removed into microtubes, which were heated for 10 min at 95°C prior to storing them at -80°C until use. In experiments that involved labelling polypeptides during the CF exposures, cells were incubated with 400 μ Ci/mL of 35 S-methionine 30 min prior to the addition of the CF, which was added at 0, 10, 20, 30, 60 and 120 min. Following these exposures, samples were processed as above and PAGE analysis as in section 2.5.1. To detect radiolabelled polypeptides, the protein gels were dried and sprayed with a fluorometric enhancer (EnHance) which increases the detection of the radiation. Alternatively, gels were incubated for 30min in EnHance solution after destaining. The dried gels were exposed to autoradiographic film at -80°C for varying lengths of time to achieve optimal band resolution and then developed and photographed.

2.6 DNA Integrity Assays

2.6.1 Extraction and Purification

General protocols for the extraction and partial purification were from Maniatis and

colleagues (1982). The size and pattern of fragments of cell DNA was assessed before and during exposure to BT. Insect and human cells were lysed with either two % (w/v) sodium dodecyl sulfate (SDS) in Tris-EDTA buffer (TE; 10 mM Tris and 1 mM EDTA, pH 8.0) or Laemmli sample buffer (see section 2.5.1). The crude cell extract was diluted 4X with TE buffer and then incubated with 100 µg/mL (final) proteinase K for two h at 37°C. This lysate was either used directly for electrophoresis or further processed by extraction of the lysate with phenol:chloroform:isoamyl alcohol (25:24:1) using three repeated treatments. Following extraction, the aqueous DNA phase was mixed with 2.5 volumes of 95% (v/v) ethanol (one h at -20°C) and the DNA precipitate was collected by centrifugation at 8000 x G for 20 min. DNA pellets were rinsed with 70% (v/v) ethanol and resuspended in TE buffer. An aliquot of the extracted DNA was mixed with 1/10 volume of 10X DNA sample buffer (20 % (w/v) Ficoll 400, 0.1M EDTA pH 8.0, one % (w/v) SDS, 0.25 % (w/v) bromophenol blue).

To determine if insect cells could form nucleosomal fragments, isolated nuclei were digested with micrococcal nuclease. To do this, T80 flasks containing confluent monolayers of insect cells were drained of media and rinsed with five mL PBS before scraping the surface with sterile cell scrapers (in five mL PBS). Cells were collected at 1000 x G for 10 min and resuspended with one mL of 0.9 % NaCl (w/v) in TE buffer. To lyse the cells, nine mL of 10 mM Tris was added and the suspension was gently mixed and vortexed (3 pulses for 5 sec each). Tubes were centrifuged for 10 min at 5000 x G and the supernatants were discarded. Each nuclear pellet was suspended in one mL of 10 mM Tris, pH 7.5 and CaCl₂ added to five mM. After removing 100 µL as a control, five units of micrococcal nuclease were added, mixed and 100 µL aliquots removed at 0, 7, 15, 30, 60, 75, 90, 105, 120 min (at RT). Each

sample was placed into a separate tube containing 100 μ L of 2X Laemmli buffer, 2.4 % (w/v) SDS and 50 mM EDTA. Samples were then treated as in section 2.6.2.

2.6.2 Agarose Gel Electrophoresis (AGE)

Following extraction and purification, DNA samples were analyzed using a submarine gel apparatus. DNA samples (10 μ g) were loaded into wells of agarose gels (50 x 1.5 mm) made with 0.8, 1.0 or 1.5 % (w/v) agarose in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate, two mM EDTA pH 8.0) $\pm$ 0.5 μ g/mL ethidium bromide (EtBr). Gels were run at 50 V until the bromophenol blue dye-front migrated at least four cm. The gel was removed from the apparatus, incubated for 20 min with 0.5 μ g/mL of ethidium bromide (if not included in the gel) and photographed using a Polaroid camera over a standard ultraviolet light (302 nm) box. Fluorescing bands were quantified by scanning photographs into imaging software (Gelbase/Gelblot), and analyzed using Microsoft Excel (version 5.0) and SlideWrite Plus software.

3. RESULTS

3.1 Characterization of Commercial BT Products

Of the five commercial BT products examined in this thesis, four are derived from subspecies *kurstaki* (*Btk*) and one from *israelensis* (*Bti*). Some of the properties of these products are given in Table 2. Only Dipel WP, was supplied as a wettable powder. This was mixed with ddH₂O to give an equivalent I.U. content to that of another product, Thuricide. All of the products examined were very viscous, with the appearance of thick chocolate milk, except Thuricide, which was tinted green. Vectobac (from *Bti*) was very similar in visual appearance to other Abbott products (Foray 48B and 76B). The most potent of the *Btk* products according to I.U. content was Foray 76B; it is also slightly more viscous than the other products due to an increased amount of particulate matter.

Examination of BT products by phase contrast microscopy without prior dilution (Figure 3A), showed that all of them were coarse aggregates. However, when examined at higher magnification by SEM (Figure 3B), distinct subcomponents could be identified; they consisted mainly of spores, seen as short, rod-like structures and bipyramidal or round structures, the PIBs of *Btk* and *Bti* respectively. Examples of *Btk* spores and crystals are indicated with labelled arrows in Figure 3. Product morphologies at the concentrations used in the animal cell exposure studies are shown in Figure 4. At the highest dose added to 6-well plates (2800 I.U. / 10 μ L), there was no substantial aggregation. The spores were approximately 0.75 x 1.2 μ m in size (diameter x length) and the PIB crystalloids ranged in size from 0.6 x 1.4 μ m to 1.2 x 2.3 μ m for *Btk*-BT products and 0.5 μ m to 1.3 μ m in diameter for *Bti*-BT products. The intact PIBs were under-represented anywhere from 25

Figure 3. Morphology of BT products. (A) Phase contrast micrographs of various BT products following staining with crystal violet and safranine. (B) Scanning electron micrographs at low (1000X) and high (9500X) magnification of Foray 48B diluted by 10-fold. Arrows indicate a rod-shaped spore and a bipyramidal PIB crystalloid. Abbreviations: DP, Dipel; F48, Foray 48B; F76, Foray 76B; TH, Thuricide; VB, Vectobac.

PHASE CONTRAST MICROSCOPY

A

B

SCANNING ELECTRON MICROSCOPY

FORAY 48B - 1000X

FORAY 48B - 9500X

Figure 4. SEM of BT products at doses used in bioassays. Air-dried products were examined at concentrations used for insect and human cell exposures in six-well plates (0.28, 28, 2800 I.U. / 10 μ L). Abbreviations: DP, Dipel; F48, Foray 48B; F76, Foray 76B; TH, Thuricide; VB, Vectobac.

Figure 5. Polypeptides associated with BT products. Samples were whole BT products (A), and Foray 48B (B) and Vectobac (C) whole products subfractionated by centrifugation into pellet (Pell) and supernatant (SN). Each sample (3000 I.U. / lane) was separated on a 12% SDS-PAGE gel and stained with Coomassie Blue R-250. Experimental details are described in section 2.5.1. Abbreviations: D, Dipel; F48, Foray 48B; F76, Foray 76B; T, Thuricide; VB, Vectobac.

to 90% compared to spores (Table 2). Dipel had the most debris of varying shapes and sizes, and the spore and PIB counts were about half that of the other products.

SDS-PAGE was used to determine the composition of the polypeptide components associated with the different BT products. As shown in Figure 5A, all *Btk*-based products had two abundant polypeptide classes, measuring 132 to 134 kDa and 62 to 67 kDa according to molecular markers. In addition, there were variable amounts of minor polypeptides measuring from ~300 kDa down to ~10 kDa in size. The maximum possible load for the mini-PAGE analysis was determined to be 3000 I.U. / 20 μ L ($\sim 7 \times 10^6$ spores). At this load, each product had slightly different amounts of the two classes of polypeptides. However, the 132 kDa polypeptide, which corresponds to the grouping of various pro- δ -endotoxins was always present in greatest quantity. Analysis of the *Bti*-based product showed that it contained polypeptides of 27, 78 and 134 kDa as major components in order of abundance. There were also many other polypeptides in minor amounts. For all products, the dominant polypeptides were only visible when stained with Coomassie Blue. The more sensitive silver stained gels indicated a high background possibly due to the presence of many proteins in low abundance. Following fractionation of the products by centrifugation, the supernatants were found to contain only trace amounts of polypeptide according to both stains. Almost all of the proteins (>95%) could be accounted for in the pellet fraction (Figure 5 B and C).

Several tests were also conducted to confirm the presence and type of organism in BT products. Spread-plating on LB-agar media of the BT products at various dilutions indicated only one colony morphology: a greyish-white, flattened disc, consisting of concentric rings

with slightly ruffled edges. At high plating densities, these colonies often appeared as polygonal shapes. Crystal violet stained the vegetative cells of the colonies purple, hence they were gram-positive. However, the spores excluded both the crystal violet and counter stain (safranine). Other components of the BT products, the PIB crystalloids and amorphous constituents, stained only with safranine and Coomassie Blue. Vegetative cells were rarely detected in samples of the BT products by light microscopy and SEM, but it is not known whether these cells were viable. In other experiments to follow (see section 3.7 and Figure 39), growth from spores of BT products resulted in increased turbidity of culture medium only in the absence of an antibiotic, gentamicin. Observations by light microscopy confirmed that the increase in turbidity of BT products was due to accumulation of vegetative *Btk* cells.

3.2 Description of Cell Systems

Details concerning the culturing of insect and human cells are given in Table 3. Since there have been no reports describing the morphology and potential differentiation of the MG1 insect cell line, this cell line was compared to *Sf9*. Light and electron microscopic analysis (see control cells in Figures 13, 15, 16 and 19) indicated that MG1 cells, in contrast to *Sf9*, had a fibroblast-like morphology, consisting of elongated protrusions, a moderately vacuolarized cytoplasm, distinct rough endoplasmic reticula and few mitochondria. However, both *Sf9* and MG1 cells stained poorly with either alcian blue or bismarck brown, stains used to identify mucin-producing cells.

Details on growth of MG1 and *Sf9* cells in Grace's medium at 27°C are given in Figure 6 and Table 3. Both cell lines demonstrated similar growth characteristics as judged

Figure 6. Growth curves of insect and human cells based on different seeding densities. Experiments involved 12-well culture plates seeded at different cell densities at 27°C (MG1 and Sf9) or 37°C (Chang and HT29) and replenishing medium every 24 h. Cell numbers, counted within three different 1 mm² areas, were taken every 24 h until crowding prevented enumeration. At this time, cells were detached from the wells by trypsinization and counted using both Neubauer-type and Coulter cell counters. Data is expressed as the average of three counts $\pm$ standard deviation.

use of DMSO to solubilize the MTT-derived formazan (Carmichael *et al.*, 1987) and parameter improvements as described here. Absorption spectra of DMSO-solubilized MTT formazan produced by the different cell lines, showed only one peak at 503 nm. However, the 490 nm wavelength was used in order to link results with data collected earlier with other plate readers. MTT concentration and time-course experiments shown in Figure 7 revealed that maximum formazan production occurred at 2.0 h with substrate concentrations ≥ 2.4 mM MTT. At incubation times >3.5 h and concentrations >2.4 mM substrate, production of formazan was inefficient. A similar analysis was done with human cell lines. As shown for HT29 (Figure 8), production of MTT-formazan was about 9-fold greater than that of insect cells. This necessitated that the formazan be diluted with DMSO so that absorbance values would be below the upper limit of detection of the spectrophotometer (~ 3.2 at 490 nm). However, Chang, Caco-2 and Hep-G2 cells did not require dilutions, as the two-h MTT incubations gave optical density values nearly equivalent to insect cells grown at 37°C . The difference in the amount of formazan produced by insect and HT29 cells was not due to incubation temperature (27°C versus 37°C), since MG1 cells incubated at 37°C only increased its formazan output by about 30%. Like insect cells, human cells showed a decrease in formazan production at MTT concentrations >2.4 mM and incubation times >3.5 h. Using parameters such as two h with 2.4 mM MTT, the amount of formazan produced per HT29 cell was approximately constant (Figure 8B).

Further modification to these assays was made so they could be used for microbial interaction studies. A background subtraction step was introduced prior to MTT incubation to reduce turbidity contributions due to BT product or *Bt* spore germination and growth at

Figure 7. Optimization of the MTT bioreduction assay for insect cells. (A) MTT concentration and incubation time was optimized using MG1 cells as an example. Experimental details are described in section 2.4.1. The two-h dotted line represents the endpoint for all other experiments. (B) Shows the relationship between the number of cells and the amount of formazan produced from 2.4 mM MTT. Formazan production for (A) and (B) is shown in optical density units at 490 nm. (C) Shows the effect of repeated rinses on attached control cells and cells after exposure to *Btk*-BT product (10^{-3} I.U. / insect cell for 16 h at 27°C). Following treatment, the supernatants were removed and insect cells were rinsed with PBS before solubilization in 100 μ L DMSO and measurement at 490 nm (see section 2.4.1). All data points represent the means of three determinations $\pm$ standard deviation.

Figure 8. Optimization of the MTT bioreduction assay for human cells. (A) MTT concentration and incubation time was optimized using HT29 cells as an example. Experimental details are described in section 2.4.1. The two-h dotted line represents the endpoint for all other experiments. (B) Shows the relationship between the number of cells and amount of formazan produced from 2.4 mM MTT. After two h at 37°C, the amount of DMSO-solubilized formazan (OD₄₉₀) was measured along with cell numbers in each well. Both these experiments required diluting the MTT-formazan 10-fold with DMSO prior to reading the optical density to ensure absorbance values below the upper limit of the spectrophotometer (ie. 3.2 at 490 nm). All data points shown were corrected for this dilution and represent the means of three replica experiments with standard deviation ($\pm$) calculated.

Figure 9. Optimal XTT and PMS concentrations for XTT-based bio-reduction assays. Concentrations of XTT and PMS were varied during incubation with 5×10^4 Sf9 cells / microtitre-plate well (~ 1500 cells/mm²). Readings were taken at 480 nm with a microplate spectrophotometer at 15 min intervals over a 15-h period at 27°C. The maximum formazan produced with each XTT and PMS concentration was plotted.

Figure 10. Comparison of XTT bioreduction activity of insect cells and BT product derivatives. XTT (750 μ M) $\pm$ PMS (25 μ M) was added to: MG1 cells in Grace's medium; spores (Foray 48B) in Grace's medium with 50 μ g/mL gentamicin; and *Bt* vegetative cells derived from six h pre-incubation of spores (Foray 48B) in Grace's medium at 27°C. Treatments were: Grace's medium alone (○); Grace's + XTT (●) and Grace's + XTT + PMS (□); cells (insect, spore or vegetative) alone (■); cells + XTT (Δ); and cells + XTT + PMS (▲). Measurements were recorded continuously at 480 nm with an autoreading microplate spectrophotometer.

Figure 11. Effect of electron transport chain toxins and ionophore on bioreduction. Dose-time response of MG1 cells exposed to different concentrations of: (A) 2,4-dinitrophenol (DNP) and (B) sodium azide (NaN_3). Exposures to calcium ionophore A23187 were also tested with *Sf9* (C) and HT29 cells (D). Following different exposures, MTT formazan was measured as described in section 2.4.1. All data points represent the means of three determinations $\pm$ standard deviation.

high I.U. and incubations >8 h in the absence of gentamicin. In addition, since formazan is produced from MTT by vegetative *Bt* cells (Seligy *et al.*, 1997a), PBS rinses were used to decrease the number of formazan-containing, bound bacteria prior to or immediately after the two h MTT incubation. Several trials involving light microscopy and spectrometry indicated that two rinses were sufficient to remove extracellular, *Bt*-associated formazan without appreciably changing the number of attached animal cells and their formazan contents (Figure 7C). However, as will be shown elsewhere in dose-response studies (see sections 3.4.1 and 3.5.1), background from BT at high doses cannot be completely eliminated.

Another tetrazolium-based indicator considered for use was XTT. Absorption spectra of the XTT formazan produced from both insect and human cells have only a single peak at 480 nm. This maximum was used for measurement of XTT formazan produced in all assays. The optimum concentrations of XTT and PMS electron coupler are derived in Figure 9. XTT was reduced by both insect and human cells, however, low amounts of PMS (>10 μ M) were necessary for outputs equivalent to MTT. In all assays that follow, 750 μ M of XTT and 25 μ M of PMS were used. The three graphs shown in Figure 10 compare the formazan production by MG1 cells, spores and vegetatively growing *Bt* cells. At equivalent numbers of cells, MG1 cells produced 10% of the XTT-formazan of vegetative *Bt* cells. *Bt* spores also reduced XTT, but only at low levels, and in the presence of PMS. Background XTT-formazan produced by Grace's and DMEM was very low in absence of PMS, but PMS-induced backgrounds were high and required subtraction from cellular XTT-formazan production.

3.3.2 Effects of Chemical Toxins

In an attempt to systematically calibrate the MTT bioreduction assays, insect cells were exposed to various cell perturbing agents. Results from exposure to the mitochondrial uncoupler 2,4-dinitrophenol, and inhibitor sodium azide, are shown in Figures 11 A and B, respectively. Both gave a 40 to 45% inhibition in formazan production over a 24-h exposure, but neither of these agents actually caused cells to detach from the well surfaces. When *Sf9* or HT29 cells were treated for 1 to 4 h with calcium ionophore A23187 at concentrations from 0.5 to 5.0 μM , formazan production actually increased by 30 to 55% compared to control cells treated with DMSO diluent alone (Figure 11 C and D). At higher doses (up to 50 μM) and exposure times (up to 24 h), formazan levels dropped by 75 to 85% of control cells and microscopical observation showed membrane blebbing. However, no cell detachment was apparent. Replating of these cells resulted in no new growth.

3.4 Insect Cell Exposures to BT Products

3.4.1 Bioreduction Assays and Morphology Changes, 24-h Endpoint

The changes in formazan production of MG1 and *Sf9* cells after a 24-h exposure to different BT products and doses, in the absence or presence of gentamicin are summarized in Figure 12. Both cell lines exhibited similar dose-response changes. When antibiotic was present, their dose-responses were at least four times less than when antibiotic was not present. At doses $>10^{-4}$ I.U./cell, formazan production progressively increased. On examination by phase contrast microscopy (see Figure 13 and also section 3.4.2), it was seen that this increase in bioreduction was not due to insect cells as they were rounded and

Figure 13. Phase contrast microscopy of MG1 after MTT bioreduction assay. MG1 cells were exposed to Foray 48B for 24 h at 27°C at the different doses indicated at the left in I.U./cell. Each well was subjected to two successive 5 sec rinses with PBS to remove excess bacteria before photography.

exhibited no *in situ* formazan deposits. The formazan was associated with vegetative *Bt* cells which were attached to the surfaces of the insect cells and at the well edges. Additional experiments using larger wells permitted more effective rinses, resulting in a marked reduction in the amount of formazan-bound vegetative cells trapped along well edges. Using these “scaled-up” assays, it was found that all doses were 100% lethal. Further testing at doses $\leq 10^{-9}$ I.U./cell gave sporadic results in that they were either 100% lethal or zero, signifying no presence of spores.

3.4.2 Kinetic Studies

Since the 24-h endpoint assays demonstrated that all doses were lethal as long as at least one spore was present to produce vegetative cells, it was necessary to determine the duration of exposure needed for dose effects to take place. This was done by determining the time necessary for 50% of the cells (relative to controls) to die or decrease their bio-reduction activity (ie. their exposure half-life). The results from a series of kinetic (dose and time) assays using both *Btk* and *Bti* -based BT products are shown in Figure 14. Based on response curves in Figures 14 A to D, half-lives were estimated and plotted as in Figures 14 E and F. The plots show that both BT products cause toxic responses in a dose-dependent manner. The response begins at about 4 h of exposure. Cross comparisons between *Sf9* and MG1 cells, revealed that MG1 maybe the most sensitive to BT.

Using phase contrast microscopy to monitor morphology changes (Figure 15), it was found that both MG1 and *Sf9* exhibited a dose-response as early as 4 h (Figure 15B). The response began with an increase in cell volume, followed by rounding and detachment from well surfaces by 8 to 12 h depending on dose. Some disruption of insect cell integrity was

Figure 14. MTT Bioreduction kinetics of MG1 and *Sf9* exposed to BT products. Monolayers were exposed to 10^{-7} (○), 10^{-5} (□) or 10^{-3} (△) I.U. / cell of Foray 48B (A, B) or Vectobac (C, D) and incubated at 27°C for the times indicated. Formazan was measured as described in section 2.4.1 and expressed as a percentage of that produced by control cells at the corresponding time points. The vertical solid lines show times where formazan levels corresponded to 50% of the control cells. Half-life values were determined from data in A to D and plotted as a function of Foray 48B (E) and Vectobac (F) dose. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 15. Phase contrast microscopy of MG1 exposed to *Btk*-BT product. (A-D) Cell monolayers were treated at 27°C as indicated. Following exposures, supernatants which included any shed cells were removed. The cells remaining attached to wells were rinsed twice with PBS and photographed (see section 2.3.1). The BT product illustrated here was Thuricide®.

MG1 - PHASE CONTRAST

Figure 16. Morphology by SEM of MG1 cells exposed to *Btk*-BT product. MG1 cell monolayers were treated at 27°C and processed for SEM (see section 2.3.2). Micrographs are representative examples of the morphologies observed. The BT product illustrated here was Foray 48B®.

Figure 17. Morphology by SEM of *Sf9* cells during exposure to *Btk*-BT product. *Sf9* cell monolayers were treated at 27°C and processed for SEM (see section 2.3.2). Micrographs are representative examples of the morphologies observed. The BT product illustrated here was Foray 48B®.

Sf9 - SEM

Figure 18. Changes in MG1 and *Sy9* cell adhesion during exposure to *Btk*-BT product. Cell monolayers were exposed to 10^{-7} (○, ●), 10^{-5} (□, ■) or 10^{-3} (Δ, ▲) I.U. /cell for 24 h at 27°C either with or without antibiotic. Following exposures for the indicated times, supernatants were removed and the number of shed cells (●, ■, ▲) was enumerated. The cells remaining attached after rinsing three times with PBS were also enumerated (○, □, Δ).

also apparent. For example, at 12 h exposure to 10^{-3} I.U./cell, few cells remained attached and these were reduced in size by ~30%. Vegetative *Bt* cells were present as early as 4 h with a steady build-up to near saturated levels by 18 h. Changes in insect cell morphology were also examined by SEM (Figures 16 and 17). Most unexposed MG1 and *Sf9* cells (>90%) appeared as spherical aggregates with rough cell surfaces. However, by 12 h of BT exposure, most cells had lost shape and integrity. The number of cells remaining attached following two rinses, were much fewer than PBS-treated cells (see Figure 18 for cell enumerations). Most *Sf9* cells (>90%) showed extensive degradation of membranes and cytoarchitecture. Nuclei that were either released as a result of cell degradation or post-treatment were generally intact. Vegetative *Btk* cells were observed attached to insect cells and to coverslip surfaces even after several rinses.

From phase contrast and scanning electron micrographs (Figures 15, 16 and 17), the 12-h BT exposures resulted in detachment of large numbers of insect cells. These experiments were also evaluated both quantitatively with cell counts (Figure 18) and qualitatively by TEM (Figure 19). In the presence of gentamicin, the amount of cell shedding was again comparable to control cells. Further tests with shed cells from either control cultures or BT product exposures with gentamicin, showed that they did not reattach or proliferate when reseeded into fresh medium at normal plating densities. Under identical conditions, without gentamicin, target cell shedding began at ~4 h exposure, with peak losses at ~12 h. The latter time interval coincides with when *Btk* vegetative cell production was readily seen. Comparison of attached and detached cells showed that most shed cells were degraded (Figure 19) and that the intact shed cells were significantly unlike trypsin-released

cells and exhibited nuclear pyknosis, cytoarchitectural disruptions and vacuolar bodies (Figure 19B).

3.4.3 Immunodetection of Protein Indicators

Several candidate antibody probes for different proteins were assessed for specificity to both human and insect cell polypeptides. Their properties are listed in Table 4. Most of these probes did not react to insect cell proteins. However, those for actin and hsp70 were deemed useful. The comparative tests with human HT29 colonic epithelial, Chang liver and K-562 and HL-60 leukaemic cells indicated that these immunoprobes recognized products with relative masses expected for actin (42 kDa) and hsp70 members (72 and 73 kDa). These same sized products were also detected in total proteins of MG1 and *Sf9* cells. Probe sensitivity tests (cell-associated antigen concentration vs antibody dilution) indicated that these antigens were reliably detected using 10 or more cells in dot-blot configuration or lysate from 5×10^4 cells (loaded per PAGE gel lane) used in Western blot analysis. In a separate study, no cross-reaction was observed with proteins from either the BT products or vegetative *Bt* cultures, and exoproteins derived from them. The abundance of the MG1 and *Sf9* cell antigens before and following *Btk*-BT exposure are shown in Figures 20 and 21. Both 72 and 73 kDa hsp70-related polypeptides were present in unexposed cells. When antibiotic was included during *Bt* product exposure, little or no change in actin or hsp70 indicators was observed (Figure 20 and 21, B and F). However, these markers began to decrease between eight and 12 h of BT exposure in the absence of antibiotic (Figure 20 and 21 A and E). Cells that were shed during BT product exposure had associated actin and at least one hsp70 member (Figure 20 and 21 C and G). The hsp73 was tentatively identified

Figure 20. Immunodetection of MG1 actin and heat shock protein hsp70. Actin and hsp70 were detected in electrophoretically separated proteins of MG1 cells that had remained attached (A, B) or were shed (C, D) following the example exposure of 10^{-3} I.U. *Btk*-BT product / cell at 27°C. Experiments were conducted in either the absence (A, C) or presence (B, D) of gentamicin. Graphs E to H correspond to Figures A to D and show densitometry measurements of actin ($\square$), hsp72 ($\bullet$) and hsp73 ($\circ$) levels relative to those of control cells.

Figure 21. Immunodetection of *Sf9* actin and heat shock protein hsp70. Actin and hsp70 were detected in electrophoretically separated total protein of *Sf9* cells that had remained attached (A, B) or were shed (C, D) following exposures to 10^{-3} I.U. *Btk*-BT product / cell at 27°C. Experiments were conducted in either the absence (A, C) or presence (B, D) of gentamicin. Graphs E to H correspond to Figures A to D and show densitometry measurements of actin ($\square$), hsp72 ($\bullet$) and hsp73 ($\circ$) levels relative to those of control cells.

as that member by combining the lysates of control cells (72 and 73kDa) with those of exposed cells and measuring its enrichment in pooled lysates. It was found that if immunoprobes were co-incubated rather than added in sequence, actin levels appeared higher compared to hsp73 levels, but this finding had no effect on interpretation of results.

3.5 Human Cell Exposures to BT Products

Based on assays developed for insect cells, comparable assays were carried out using the human cell lines listed in Table 3. After 10 trial experiments, it was established that HT29 and Chang cells were the most reliable and informative. The cell lines, HL-60 and K562, can only be propagated by suspension culture, which required extra manipulations (ie. centrifugation steps). As a consequence, most experiments described in the following sections involved HT29 and Chang cells.

3.5.1 Bioreduction Assays and Morphology Changes, 24-h Endpoint

As shown in Figure 22, both HT29 and Chang cells exhibited loss of bioreduction activity comparable to that seen in insect cells (see section 3.4.1 and Figure 12) on prolonged exposure to BT products. However, in the absence of gentamicin, the 24-h exposure was too destructive and failed to show differences between doses except for those due to increased formazan production resulting from adhering bacteria.

The phase contrast micrographs of HT29 cells exposed for 24 h to the 10^{-7} I.U./cell dose in the presence of gentamicin as shown in Figure 23, resulted in little or no change in cell density or morphology. The monolayer essentially remained intact with close cell-to-cell interactions, indicating little or no disruption in cell interactions. In the absence of

Figure 22. Bio-reduction activity of HT29 and Chang cells after exposure to *Btk*-BT product. The cell monolayers were exposed to dilutions of *Btk*-BT product in the presence (●) or absence (○) of gentamicin. At 24-h exposure, the production of formazan was quantified (see section 2.4.1). The dotted line represents the outcome of the same assay using 6, 12 and 24 well plates. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 23. Phase contrast microscopy of HT29 cells exposed to *Btk*-BT product. HT29 cell monolayers were exposed to PBS (A), or 10^{-7} I.U./cell in the presence (B) or absence (C) of gentamicin. Following exposure, the wells were rinsed twice with PBS and photographed (see section 2.3.1).

gentamicin, with the same BT dose, large numbers of vegetative *Btk* were seen, but no spores or crystalloids were present. Many HT29 cells detached from the well surfaces, but those that remained attached were reduced in size and severely disrupted. Cell-to-cell interactions were also no longer apparent.

3.5.2 Kinetic Studies

From the morphological observations recorded at 24 h (Figure 23) and observations during the 24-h exposure, it was apparent that the bulk of the cell loss occurred during the first six h. The graphs in Figure 24 illustrate this through a detailed enumeration of cells during various exposure intervals up to 8 h. Exposure to PBS resulted in a loss of <10 cells/mm² (0.1 to 0.3%) from the well surface during the time period. However, with BT product present and no antibiotic, the number of attached cells remaining at 8 h was <20% of control for either HT29 or Chang. The half-life for attached cells of HT29 was 4.4 h and for Chang, it was 6.2 h. These values are about half of values obtained in insect cell exposures to BT. Also, the number of shed cells was less than expected from attached cell counts, especially in the case of Chang cells.

Since both HT29 and Chang cell-lines exhibited the most extensive morphological damage during the first four to six h of exposure, bioreduction assays were routinely conducted over six h in order to obtain half-lives for each dose. Target cell bioreduction changes resulting from Foray 48B (*Btk*) and Vectobac (*Bti*) exposure are shown in Figure 25. With gentamicin present during the exposures, both products induced little or no decrease in HT29 MTT bioreduction. The maximum responses were <6% and <10% for Foray 48B and Vectobac, respectively (Figures 25 B and D). However, the exposure results without

Figure 24. Adhesion of HT29 and Chang cells exposed to *Btk*-BT product. Cell monolayers were exposed to 10^{-3} I.U./cell for zero to eight h at 37°C with (○,□) or without (●,■) gentamicin. Following exposures, supernatants were removed and shed (□,■) and attached (○,●) cells were enumerated. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 25. Bioreduction kinetics of HT29 cells exposed to *Btk* and *Bti* -BT products. (A-D) Cell monolayers were exposed to PBS (●) or, 10^{-7} (■), 10^{-5} (▲) or 10^{-3} (◆) I.U. /cell of *Btk*-BT product (A, B) or *Bti*-BT product (C, D) at 37°C for the times indicated. The formazan produced by these cells was measured as in section 2.4.1 and expressed as a percent of control cells. The dotted line indicates 50% of control cells at which corresponding half-life values were obtained from the x-axis (E). Data points represent the means of three determinations $\pm$ standard deviation.

Figure 26. Phase contrast microscopy of HT29 exposed to *Btk*-BT product. Cell monolayers were exposed to 10^{-3} I.U./cell at 37°C for the times indicated. Following exposure, supernatants and shed cells were removed and attached cells were rinsed three times with PBS and photographed as described in section 2.3.1.

HT29 - PHASE CONTRAST

HT29 - SEM

119

NO BT EXPOSURE 6h - 500X

A

NO BT EXPOSURE 6h - 3000X

B

NO BT EXPOSURE 6h - 3000X

C

NO BT EXPOSURE 6h - 3000X

D

10^{-3} IU/CELL: 6h - 500X

E

10^{-3} IU/CELL: 6h - 3000X

F

Figure 28. Bio-reduction kinetics of Chang cells exposed to BT products. (A-D) Cell monolayers were exposed to PBS (●) or, 10^{-7} (■), 10^{-5} (▲) or 10^{-3} (◆) I.U./cell of *Btk*-BT product (A, B) or *Bti*-BT product (C, D) at 37°C for the times indicated. The formazan produced by these cells was measured as in section 2.4.1 and expressed as a percent of control cells. The dotted line indicates 50% of control cells at which corresponding half-life values were obtained from the x-axis (E). Data points represent the means of three determinations $\pm$ standard deviation.

Figure 29. Phase contrast microscopy of Chang cells exposed to *Btk*-BT product. Cell monolayers were exposed to 10^{-3} I.U./cell at 37°C and supernatants and shed cells were removed. Cells remaining attached were rinsed twice with PBS and then photographed as described in section 2.3.1. (A, B) Cells exposed to PBS. (C, D) Examples of BT exposure showing enlarged Chang cells with rafts of *Btk* vegetative cells inside them.

CHANG - MODULATION CONTRAST

NO BT EXPOSURE 6h (1000X)

A

NO BT EXPOSURE 6h (2300X)

B

10^{-3} IU/CELL 6h EXPOSURE (1000X)

C

10^{-3} IU/CELL 6h EXPOSURE (2300X)

D

Figure 30. Morphology by SEM of Chang cells exposed to *Btk*-BT product. (A, B) Typical morphologies of cells exposed to PBS alone. (C) Monolayer disruption following 6-h exposure at 37°C. (D, E) Examples of disrupted cell morphologies. (F) Example of clustered vegetative *Bt* cells in close association with a disrupted Chang cell. Experimental details are described in section 2.3.2.

CHANG - SEM

125

NO BT EXPOSURE 6h - 500X

A

NO BT EXPOSURE 6h - 3000X

B

10^{-3} IU/CELL 6h - 500X

C

10^{-3} IU/CELL 6h - 3000X

D

10^{-3} IU/CELL 6h - 3000X

E

10^{-3} IU/CELL 6h - 3000X

F

antibiotic (Figures 25 A, C and E) were comparable for the two products if spore contents for each product dose were initially made equivalent.

Detailed examinations of HT29 morphology changes occurring with BT product exposure are shown in Figures 26 and 27. The phase contrast and SEM data for Foray 48B shown here are very similar to those observed with Vectobac at equivalent spore-dose levels. Additional changes in morphology that were not apparent by phase contrast microscopy in the 24-h exposures included cell swelling and lysis at five h and volume reduction by ~50% at six h exposure (Figure 26). Comparison of SEMs of exposed and unexposed HT29 cells (Figure 27) revealed at least three different morphologies. About 80% of the control cells exhibited a raised, slightly flattened appearance with numerous microvilli, while others were either columnar in shape (~10%) or were rounded with a depression or opening at their apical surfaces (~10%) resembling a globular cell morphology (Lu *et al.*, 1992). At six h exposure to 10^{-3} I.U. /cell, the monolayers were completely disrupted and considerable debris was apparent. At 3000X magnification, none of the cells had microvilli on their surfaces and most cells were lysed or fragmented. .

Data in Figure 28 shows the bioreduction activity of Chang cells during exposure to Foray 48B (Figure 28 A and B) and Vectobac (Figure 28 C and D). Controls and BT-treated cells in the presence of gentamicin, showed no significant decreases in formazan levels over the eight h exposure interval (Figures 28 B and D). However, in the absence of antibiotic, either BT product caused extensive cell damage with half-lives between 5.8 and 6.2 h. The dose-response was almost the same for each dose (Figure 28E) in contrast to HT29 and the insect cell lines.

Figure 31. Survey of human cell lines for immunodetection of actin. (A) Western blot analysis of proteins of Chang, Hep-G2, HT29 and K562 cells that remained attached (zero to six hours), or were detached or floating (6F) following exposure to 10^{-3} I.U./cell at 37°C. (B) Shows the quantified actin levels relative to those of control cells as determined by densitometry (see section 2.5.2). The arrows intersect the x-axis at time points which correspond to where the marker levels have decreased by 50% of control cells.

ACTIN

A

B

Figure 32. Survey of human cell lines for immunodetection of heat shock proteins. Immunoprobe analysis for hsp60 (A) or hsp70 (B) of electrophoretically transferred total protein from human cells that remained attached (zero to six hours) or were detached or floating (6F) following exposure to 10^{-3} I.U./cell at 37°C. (C) Shows heat shock protein levels of BT product-treated cells relative to those of control cells as determined by densitometry (see section 2.5.2). The arrows intersect the x-axis at time points which correspond to where the marker levels have decreased by 50% of control cells.

Figure 33. Immunodetection of cyclin and glutathione-S-transferase (GST) from HT29 cells.

(A) Levels of cyclin and GST from immunoprobe analysis of electrophoretically transferred total protein from HT29 cells following exposures to 10^{-3} I.U./cell for zero to six h at 37°C.

(B) Shows the quantified marker levels relative to those of control cells as determined by densitometry (see section 2.5.2). The arrows intersect the x-axis at time points which correspond to where the marker levels have decreased by 50% of control cells.

Figure 34. Immunodetection of tubulin from human cells lines. (A) Levels of tubulin determined from Western blot analysis of total protein from HT29 and K562 cells following exposures to 10^{-3} I.U./cell from zero to six h at 37°C. (B) Shows the quantified tubulin levels relative to those of control cells as determined by densitometry (see section 2.5.2). The arrow intersect the x-axis at the time point which correspond to where the marker level has decreased by 50% of control cells.

TUBULIN

A

B

ranged between 4.2 and 5.7 h, which is consistent with the number of attached Chang and HT29 cells during BT exposure (see Figure 24). Since only two probes were the most reliable (actin and hsp70), they were the probes most often used to measure changes in cell protein levels during BT treatment. For all cells, actin levels were predictive of attached cell number (Figure 31). However, for shed cells at six h exposure, actin was not present at the levels expected based on estimated loss of attached cells. Unlike what was seen with insect cells, the hsp70 immunoprobe detected only one human cell product measuring 73 kDa (Figure 32B). By six h, this protein was no longer detected in HT29 and Hep-G2. Chang cells exhibited no detectable reaction with the hsp 70 immunoprobe. This was also found using a probe for hsp60 which was positive for HT29 and K562 cells (Figure 32A). The changes in the levels of this marker during BT product exposure were very similar to those of hsp73. Using probes for cyclin and glutathione-S-transferase, only proteins of HT29 were immunoreactive. As shown in Figure 33, the levels of cyclin decreased the most of any other marker, resulting in a 50% decrease by <2 h of exposure compared to control cells. However, changes in morphology and cell shedding were not observed until four h exposure. The decrease in levels of GST during BT exposure were comparable to those measured for hsp's as well as tubulin (Figure 34). However, the tubulin immunoprobe was weakly reactive and only useful at high concentration (1:10 dilution). At this concentration, the reductions of tubulin during BT exposures were estimated to be similar to actin for HT29 cells. As indicated in various experiments presented here, K562 proteins (actin, hsp60, hsp73, tubulin) basically remained at constant levels throughout the 6-h exposure regime. Since these cells grow only in suspension, the invariant change in protein levels suggest that K562 were

Figure 35. DNA recovered from insect cells exposed to BT. (A) DNA from cell monolayers of MG1 cells exposed to 10^{-7} (●), 10^{-5} (■) or 10^{-3} (▲) I.U./cell at 27°C for the times indicated (in hours). The DNA of these cells was extracted and separated by agarose gel electrophoresis as described in sections 2.6.1 and 2.6.2. DNA was also quantified by densitometry and plotted in relation to DNA recovered from cells exposed to PBS alone. Lanes denoted 7F, 5F and 3F correspond to DNA recovered from cells shed into the medium after treatment with doses of 10^{-7} , 10^{-5} and 10^{-3} I.U./cell, respectively. (B) MG1 and *Sf9* chromatin DNA incubated with micrococcal nuclease (0.05 units / μ g nuclear DNA) for various times (in minutes). DNA released by detergent and proteinase K treatment was separated by agarose gel electrophoresis as described in sections 2.6.1 and 2.6.2. Commercial molecular weight markers (M) range from 0.1 to 23 kilobase pairs.

Figure 36. DNA recovered from human cells exposed to BT. (A) Agarose gel electrophoresis of DNA from cell monolayers of HT29 cells exposed to 10^{-3} I.U./cell for 24 h at 37°C. The DNA from control (c), attached (a) and detached (d) cells was extracted and size fractionated by agarose gel electrophoresis as described in section 2.6.1 and 2.6.2. (B) Agarose gel electrophoresis of DNA from HT29 and Chang cells shed during exposure to 10^{-3} I.U./cell for the time periods indicated (in hours). (C) The staining intensity of DNA from shed cells was quantified by densitometry and plotted relative to DNA levels of attached control cells exposed to PBS alone. Molecular weight markers, M_r range from 0.1 to 23 kilobase pairs.

HUMAN CELL DNA

A

B

C

resembled the DNA from shed insect cells. However, the DNA from attached cells appeared as a smear or occasionally as discrete fragments, essentially multiples of approximately 160 bp (Figure 36A). In contrast, DNA from HT29 exposed to BT products for 24 h in the presence of gentamicin, showed no evidence of DNA loss or degradation. The DNA of shed cells and attached cells resulting from earlier exposure time points was also examined. These DNA samples showed evidence of degradation after 3 to 6 h exposure to BT, but no nucleosomal ladders were ever seen. Similar DNA analyses were repeated with Chang, Hep-G2, HL-60 and K562 cells which showed only a smear-type degradation pattern.

3.7 Effect of Temperature on Insect Cell Toxicity

Comparison of the toxicity patterns between insect and human cells indicated that the major difference was the time at which damage was induced. For human cells this occurred about 5 to 9 h faster than insect cells, depending on dose. One obvious difference between the two bioassays, besides cell type and culture medium, was the temperature at which the cells were maintained and exposed (27°C versus 37°C). Therefore, BT exposure experiments were done at both 27°C and 37°C, and also at a lower temperature of 23°C and an intermediate temperature of 34°C. As a control, the temperature dependency of formazan produced (O.D.₄₉₀) by either MG1 or *Sf9* was investigated. The latter results showed an increase of ~ 30% between 27°C and 37°C. However, for BT exposures, the loss in formazan production increased as both temperature and dose increased (Figure 37). By calculating the half-life obtained from such experiments, dose effects increased directly as temperature increased. At 37°C there was little difference in 10⁻⁵ and 10⁻³ I.U./cell doses.

Figure 37. Bio-reduction assay of MG1 and *Sf9* cells exposed to *Btk*-BT product at various temperatures. A and B show typical data for MTT formazan production over the course of 24 h at various temperatures using the highest dose of Foray 48B (10^{-3} I.U./cell). The dotted line intersects the point at which formazan levels have decreased by 50% of control cells (half-life). C and D show derived half-lives for formazan production based on control cells and data in A and B. Data points represent the means of three determinations $\pm$ standard deviation. The standard deviation values were very small.

Figure 38. Summary of actin immunoprobe analysis of insect cells exposed to *Btk*-BT product at various temperatures. (A) Example of an actin immunoblot of total proteins from attached MG1 cells exposed to different doses, 10^{-7} ($\circ$), 10^{-5} (Δ) and 10^{-3} ($\square$) I.U./cell (indicated as -7, -5 and -3, respectively in A), temperatures (23, 27, 34 and 37°C) and times (zero to 16 h). (B) Quantification of actin from blots in (A). Data points represent the means of three determinations $\pm$ standard deviation. (C) Estimates of actin half-life expressed in hours for 10^{-7} ($\circ$), 10^{-5} (Δ) and 10^{-3} ($\square$) I.U./cell as a function of assay temperature.

Figure 39. Effect of temperature on vegetative *Btk* proliferation. (A) Cell production in Grace's medium at specified temperatures and doses using an automated reading system (wavelengths of 450 nm and also 600 nm (not shown)). (B) Estimated total *Btk* cell production generated at 20 h growth at the different temperatures and doses.

Figure 40. Relationship of actin half-life of BT-exposed insect cells and *Btk* proliferation. Data for *Bt* cell production was obtained from experiments described in Figure 39A and plotted against MG1 (A, B and C) and *Sf9* (D, E and F) cell half-life information from Figure 38C. The line of best fit from these exposures, and their regressions (r^2), probability values (p), slopes (m) and sample number (n) are shown for 10^{-7} I.U./cell (A, D), 10^{-5} I.U./cell (B, E) and 10^{-3} I.U./cell (C, F) doses.

A summary of several experiments using actin as an indicator, are shown in Figure 38. Quantification of the actin abundance after each exposure (Figure 38B) agreed with the results from formazan assays. The actin of control cells remained relatively constant over the same temperature regime. On BT exposure, the actin half-life was inversely proportional to dose and temperature.

Further investigations conducted to establish temperature effects on spores (Figure 39) showed that in absence of insect cells, *Btk* spores produced growth curves consistent with temperature effects in BT exposure experiments. Also, the bacterial cell output at 34°C and 37°C was about twice that at 23°C and 27°C (Figure 39B). The comparison of bacterial cell production with corresponding actin half-life measurements (Figure 40) established that: (1) the temperature effect was relatively independent of insect cell type (MG1 or *Sf9*) as demonstrated by similar slopes of “best-fit” lines; (2) doses spanning five orders of magnitude were least clustered at the lowest temperatures and; (3) at 34°C and 37°C, different doses produced similar levels of cytotoxicity, which were close to that for BT exposures to human cells (4 to 6 h) (Figures 25, 28, 31 and 32). These results demonstrate that toxicity is dependent on the rate of *Bt* cell production, which is affected by incubation (exposure) time, dose and temperature. Numerous experiments were also carried out with DMEM medium and BT alone. These studies showed little or no *Bt* growth without human cells present, but when human cells were present, temperature governed *Bt* growth and toxicity.

3.8 Cytotoxicity of BT Product Subcomponents

3.8.1 Comparative Analysis of Liquid and Coarse Particulate Fractions

To determine what BT subcomponent(s) contributed most to cytotoxicity, BT products were subfractionated by centrifugation into a particulate-rich pellet (containing spores and PIB-associated δ -endotoxin) and a particulate-free supernatant. Time-course exposures were then carried out (Figure 41). MG1 and HT29 cell lines were affected most by either whole product or pellet when they were made equivalent to 10^{-3} I.U./cell. In the presence of antibiotic, neither BT product nor its subfractions affected the cell lines much. To determine if proteins associated with the exterior of spores were responsible for toxicity, both the pellets and supernatants were extensively digested with a broad-spectrum protease (proteinase K), and spores were retrieved and used for exposure of MG1 or HT29 cells. Severe damage resulted only with the spore fraction in the absence of antibiotic.

A summary of results obtained from analyses conducted with concentrated BT product liquid fraction and filtrates of it, is given in Figures 42 and 43. The polypeptide profile of a 10X enrichment with antibiotic or after filtration were the same. The filtrates, even if concentrated by 10X, contained relatively low amounts of protein (Figure 42A), only 0.5 to 5 $\mu\text{g/mL}$ or 0.001 to 0.02% of the total protein in BT products. In terms of amount of definable proteins, Vectobac and Dipel had the least and Foray 48B had the most. The polypeptide profiles of Foray 48B and 76B were almost identical, except that Foray 76B had less total protein (~30% of Foray 48B). The most abundant polypeptides of Thuricide measured 20 kDa and 75 kDa, whereas Foray products had polypeptides of 17, 25, 35 and 75 kDa. Comparison of bioreduction assay results in Figure 42 B show similar decreases in

Figure 41. Bioreduction activity of MG1 and HT29 exposed to *Btk*-BT product or subcomponents. Cell monolayers were exposed to dilutions equivalent to 10^{-3} I.U./cell using either whole Foray 48B, Foray 48B supernatant (F48 SN), Foray 48B pellet (F48 PEL) or 48B PEL and 48B SN (data not shown) treated with proteinase K. Exposures were conducted for the times indicated in the absence and presence of gentamicin. The formazan produced by these cells was measured as described in section 2.4.1 and expressed as a percentage of control cell activity. The dotted lines represent the outcome of the same assays using 6-well plates. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 42. Protein content and cytolytic activity of BT product supernatants. (A) Coomassie-stained SDS-PAGE gel of filtered (0.45 μ m) and 10-fold concentrated supernatants from commercial BT products (Dipel, D; Thuricide, T; Foray 48B, F48; Foray 76B, F76; and Vectobac, VB). Lanes contain ten μ L of each concentrate. (B) MTT bioreduction activity of MG1 and HT29 cells exposed to dilutions of *Btk*-BT product filtrate for 24 h at either 27°C or 37°C. The bioreduction changes from exposure to all *Btk*-based BT product filtrates were almost super-imposable with that of Foray 48B filtrate shown as an example. Data points represent the means of three determinations $\pm$ standard deviation. (C, D) Phase contrast micrographs of MG1 (C) and HT29 (D) exposed for 24 h to PBS (control) or formulation filtrate (FF) equivalent to a dose of 1.27 I.U./cell Foray 48B which was derived by diluting the filtrate 10-fold. Note that within two h, MG1 cells become sharp and angular in shape. At the same time, HT29 monolayers are disrupted with large spaces between cells which are elongated and irregular in shape.

Figure 43. Bio-reduction activity of insect and human cells exposed to *Bti*-BT product supernatants. (A) MTT formazan production of insect (MG1 and *Sf9*) and human (HT29 and Chang) cells during exposures to 0, 0.25, 0.5, 1, 2.5, 5, 10 % (○, ●, □, ■, △, ▲, ◇, respectively) Vectobac product filtrates for the times indicated at 27°C (insect cells) or 37°C (human cells). (B) Multiwell dish demonstrating the trypsin (TRYP) -like cell detachment of HT29 cells at two h exposure to dilutions of Vectobac formulation filtrate (FF).

B

A

activity with MG1 and HT29 cells after exposure to Foray 48B filtrates, but only at concentrations of 0.1X (equivalent to 1.27 I.U./cell), which is 1,270-fold greater than the highest dose generally used in BT product experiments. Comparisons of phase contrast micrographs of cells exposed to either PBS or 10-fold dilutions of Foray 48B filtrates (Figures 42 C and D), revealed that the cells remained attached, but did not resemble control cells in that they had lost their regular rounded appearance as well as their ability to produce *in situ* deposits of MTT formazan. These morphological changes were notably different from the induced cellular damage observed in exposures to whole BT product or proteinase K treated spores.

Similar exposure experiments with concentrated Vectobac filtrates showed that MG1 and S9 cells were inhibited in bioreduction by 50% at 4 h and 3 h respectively (Figure 43A), whereas HT29 and Chang cells reached the same point in less than two h. However, the latter response was due to complete detachment of all cells during the rinses of the MTT assay. For all cell lines tested, the actual formazan production by these detached cells remained comparable to PBS-treated cells. A comparison of HT29 cells treated with either the Vectobac filtrate or trypsin is shown in Figure 43B. Both exposures resulted in similar types of cell detachment rates when trypsin was used at a concentration of 0.25% (v/v). However, this activity was not seen when the Vectobac supernatant was made equivalent to 10^{-7} to 10^{-3} I.U. / HT29 cell, the dose range used most in this research.

3.8.2 Paraspore Inclusion Bodies (PIBs)

Products Foray 48B and Vectobac were used to obtain PIBs. The method of enrichment is summarized in steps A to D of Figures 44 and 45. Foray 48B produced five

Figure 44. Characterization of sucrose gradient-enriched PIB from Foray 48B[®]. (A to D) Show steps in the preparation of PIB and solubilized “activated” δ -endotoxin. (E) Coomassie blue stained polypeptides of 10 μ L of each fraction separated by SDS-PAGE. (F and G) Scanning electron micrographs of the F4P1 fraction prior to (F) or after Na_2CO_3 and trypsin treatment (G). Phase contrast microscopy was also used to verify contents of each fraction. Following trypsin treatment, F4S2 was rich in 67 kDa polypeptide, but nothing was seen by SEM.

Figure 45. Characterization of sucrose gradient-enriched PIB from VectoBac 12AS®. (A to D) Show steps in the preparation of PIB and solubilized “activated” δ -endotoxin. (E) Shows Coomassie blue stained polypeptides of 20 μ L of each fraction separated by SDS-PAGE. (F and G) Scanning electron micrographs of the V3 fraction prior to (F) or after Na_2CO_3 and trypsin treatment (G). Phase contrast microscopy was also used to verify contents of each fraction. Following trypsin treatment, V3S2 contained both 22 and 27 kDa polypeptides, but nothing was seen by SEM.

Table 6. Fraction Contents of BT Products Separated Using Sucrose Gradients

Sucrose Gradient Fraction	Spore Number (per mL) ^a	PIB Number (per mL) ^a	Protein Content (mg/mL) ^b
Foray 48B (whole)	3.2×10^{10}	1.4×10^{10}	57
F1	1.2×10^4	1.1×10^4	0.03
F2	1.6×10^4	2.3×10^6	0.21
F3	4.1×10^8	1.1×10^9	0.70
F4	1.4×10^8	1.3×10^9	0.68
F5	6.1×10^9	4.2×10^4	0.13
Vectobac (whole)	2.9×10^{10}	1.0×10^{10}	32
V1	8.0×10^3	7.2×10^3	0.03
V2	1.7×10^5	1.2×10^3	0.05
V3	4.4×10^8	1.7×10^8	0.18
V4	4.0×10^9	4.2×10^4	0.11

^a These values were determined by diluting samples and enumerating under a phase contrast microscope.

^b Concentrations of each sample were based on Coomassie G-250 staining of BSA.

^c Data from Seligy *et al.*, 1997a.

Figure 46. Bioreduction activity of *Sy9* exposed to PIB. Exposures were conducted at 27°C for either two h (A) or four h (B) with PIB prepared as described in Figures 44 and 45. Following exposure, formazan content was determined as described in section 2.4.1. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 47. Bio reduction activity of HT29 exposed to PIB. Exposures were conducted at 37°C for either two h (A) or four h (B) with PIB prepared as described in Figures 45 and 46. Following exposure, formazan content was determined as described in section 2.4.1. Data points represent the means of three determinations $\pm$ standard deviation.

A**2HR ASSAY** **B****4HR ASSAY**

CONTROL 500X

CONTROL 3000X

F48 PIB - 24h - 500X

F48 PIB - 24h - 3000X

VB PIB - 24h - 500X

VB PIB - 24h - 3000X

Figure 50. SEM of HT29 exposed to PIB. (A, B) Typical control-cell morphologies after exposure to PBS for 24 h at 37°C. (C, D) Cells following exposure to 100 µg/mL Foray 48 PIB for 24 h at 37°C. (E, F) Cells following exposure to 100 µg/mL Vectobac 48 PIB for 24 h at 37°C. Experimental details are described in section 2.3.2.

fractions (F1-F5) after centrifugation. Polypeptide analysis of the fractions revealed that most of the 132 and 67 kDa proteins were in F3 and F4 (Figure 44E). However, phase contrast microscopy revealed that fraction F3 had about three times more spores than F4, whereas the last fraction (pellet; F5) consisted mainly of spores (~90%) with some PIB structures. Micrographs of fraction F4 prior to (Figure 44F) or following (Figure 44G) adjustment of pH to 10 and addition of protease revealed that treatment resulted in the presence of only spores, suggesting that the crystals had been solubilized. When supernatants from this treatment were examined by SEM, no particulates were visible (data not shown).

A similar sucrose step gradient separation of *Vectobac* (Figure 45) gave four fractions with distinct SDS-PAGE and SEM patterns. As shown in Figure 45E, the highest concentration of polypeptides was in gradient fraction V3. Treatment of V3 at pH 10 and protease digestion resulted in a decrease of the 132 kDa polypeptide and the appearance of a 22 kDa polypeptide (V3S1). Following centrifugation, the supernatant (V3S2) contained approximately 30% of the total proteins, all of the 22 kDa product and about 90% less of the 27 kDa polypeptide, compared to fraction V3. The SEM analysis revealed that fractions V1 and V2 contained amorphous structures that varied in shape and size, but did not resemble spores, but fraction V2 contained some *Bti* PIB (data not shown). The majority of these spherical shaped crystalloids ($0.6 \mu\text{m} \pm 14\%$ diameter) were in fraction V3 and harvested as V3P1. The pellet of fraction V4 contained both spores and PIB structures (data not shown). When fraction V3P1 was resuspended with a buffer of pH 10 and protease, there was a notable reduction in the number of the crystals after an incubation of one h at 37°C. However, some PIB still remained as indicated by analysis of V3P2 (Figure 45G). The V3S2

recovered by centrifugation and dialysis had no particulate material.

Sucrose gradient fractions were quantified for spore and PIB, and protein content using BSA as a standard (Table 6). Spore numbers were reduced by 80 to 85% after the first two sucrose gradient separations and were always greatest in the pellet fractions. PIBs were always most numerous in the penultimate fractions (F4 and V3) although large numbers of spores were also present. These spores could be separated from the PIB material by solubilization and centrifugation as described in Figures 44D and 45D.

Prior to enrichment and solubilization, Foray 48B and Vectobac products contained 57 and 32 mg/mL total protein, respectively. Following fractionation, the recovered protein associated with PIB-enriched fractions F4 and V3, was $\leq 1\%$ of the total protein of BT products (0.68 mg/mL and 0.18 mg/mL, respectively) (Table 6). This is at least 18X the amount of protein in fractions treated with dH₂O ($< 10 \mu\text{g/mL}$).

The cytotoxicity of F4 and V3, and solubilized fractions F4S2 and V3S2 was determined by the MTT bioreduction assay using whole BT product as standard with two and four h exposures. As shown in Figures 46 A and B for *Sf9* cells, neither carbonate buffer nor trypsin caused a decrease in formazan production. In exposures to Foray 48B PIB, there was a low level of cytotoxicity ($< 10\%$) with doses between 6 and 100 $\mu\text{g/mL}$ for both the two and four h assays. In contrast, similar treatment with Vectobac PIB caused a large decrease in formazan production with an LD₅₀ of $\sim 55 \mu\text{g/mL}$ for the two h assay and $\sim 38 \mu\text{g/mL}$ for the four h assay. Similar experiments with HT29 cells shown in Figures 47 A and B indicated that HT29 cells were more sensitive to solubilized Foray PIB exposures than *Sf9* cells, but exposure to solubilized Vectobac PIB resulted in comparable LD₅₀s to those measured for

Sf9.

Further experiments involving the measurement of actin levels and cell attachment of *Sf9* and HT29 exposed for four h to different doses of Foray 48B PIB and Vectobac PIB were carried out. Results shown in Figure 48 are in agreement with the changes in formazan production of these cells. The Vectobac PIB was again more toxic than Foray 48B PIB. Even at the highest dose of Foray PIB (100 µg/mL), no major reductions in actin or cell number was observed for either cell line. At the highest dose of Vectobac PIB, actin was reduced by ~27% in *Sf9* cells and ~40% in HT29 cells, and the number of cells lost was about the same.

Scanning electron micrographs of *Sf9* and HT29 exposed to the highest dose (100 µg/mL) of solubilized Foray 48B PIB and Vectobac PIB are shown in Figures 49 and 50. They reveal that approximately 6% of *Sf9* exposed to Foray 48B PIB were larger in size by ~5.6 fold (ie. estimated volume). In treatments with an equivalent amount of Vectobac PIB, ~67% of cells exhibited a volume increase. In contrast, HT29 showed no changes in cell morphology, or volume (Figure 50).

3.9 Toxicity Associated with Vegetative Cell Production

3.9.1 Washed Vegetative *Btk* Cells

Experiments from sections 3.7 and 3.8.1, revealed that toxicity to either insect or human cells was greatest when spores were permitted to germinate and produce vegetative cells. Based on equivalent number of vegetative cells, this toxic response was the same for both *Btk* and *Bti*.

The ability of vegetative cells to cause insect and human cell damage was investigated in greater detail. Since it was found that vegetative cells were capable of bioreducing MTT and therefore distorting target cell responses, an alternate approach using protein (ie. actin and hsp70) assays was taken. As a starting point, MG1 cells were treated with vegetative *Bt* derived from 6 h incubation at either 27°C or 37°C in either LB broth or Grace's medium. After production of the *Bt* cultures, a centrifugation step was carried out to obtain pellets and supernatants for testing. The pellets were rinsed with fresh media and the number of vegetative cells was determined and adjusted to 2.4×10^6 cfu/mL. This value is equivalent to the number of spores in the 10^{-3} I.U./cell dose of BT product tested earlier. From results shown in Figure 51, it was determined that toxicities of *Bt* vegetative cells grown at the two different temperatures were essentially comparable and detectable at ≥ 4 h of exposure. In experiments with HT29 and Chang cells (Figures 52 A and B), changes in actin levels from attached cells took place at ≥ 1.5 h. But, if gentamicin was introduced at ≤ 1 h into the exposure regime, cytotoxicity was prevented. In experiments with unwashed vegetative cells, the exposure experiments showed marker losses within 10 min with all target cell systems, suggesting that toxicity was loosely associated with vegetative cells. Identical assays with the supernatant fraction (ie. cell-free conditioned medium) revealed that it contained the bulk of the cytolytic activity (see section 3.10.1 for more details).

3.9.2 *Bacillus subtilis* and *Escherichia coli*

B. subtilis and *E. coli* were exposed to insect and human cells to determine if they produced cytotoxic effects comparable to BT products or vegetative *Bt* cells. Results in Figure 53 show MTT bioreduction assays of S9 and HT29 cells exposed to either bacteria

Figure 52. Immunodetection of actin from HT29 and Chang exposed to vegetative *Btk* cells. Blots were performed as in section 2.5.2, using HT29 (A) and Chang (B) cells following exposure to vegetative *Btk* derived from growth of Foray 48B in DMEM medium for six h at 37°C. Doses of *Btk* were made equivalent to 10^{-3} I.U./cell based on *Btk*-BT product cfu counts (~2400 cfu/I.U.).

Figure 53. Bioreduction activity of Sf9 and HT29 exposed to either *Bacillus subtilis* or *Escherichia coli*. Animal cells were prepared in 96-well plates and exposed to either bacterial species for 24 h in media with (●, ■) or without (○, □) gentamicin at doses equivalent in cfu content to low (L), medium (M) and high (H) doses of Foray 48B.

Figure 54. Effect of *Bacillus subtilis* or *Escherichia coli* Exposure on Sf9 and HT29 Actin Levels. Animal cells were prepared in 96-well plates and exposed to either bacterial species for 24 h in media and exposed with (A) or without (B) gentamicin at doses equivalent in cfu content to low (10^{-7} I.U./cell), medium (10^{-5} I.U./cell), and high (10^{-3} I.U./cell) doses of Foray 48B.

Figure 55. Effect of *Bacillus subtilis* or *Escherichia coli* on morphology of Sf9. (A, B) Typical control-cell morphologies after exposure to PBS for 24 h at 27°C. (C, D) Cells following exposure to *B. subtilis* for 24 h at 27°C. (E, F) Cells following exposure to *E. coli* for 24 h at 27°C at doses equivalent in cfu to high BT dose. Experimental details are described in section 2.3.2.

CONTROL 500X

A

CONTROL 3000X

B

B. subtilis - 24h - 500X

C

B. subtilis - 24h - 3000X

D

E. coli - 24h - 500X

E

E. coli - 24h - 3000X

F

Figure 56. Effect of *Bacillus subtilis* or *Escherichia coli* on Morphology of HT29. (A, B) Typical control-cell morphologies after exposure to PBS for 24 h at 37°C. (C, D) Cells following exposure to *B. subtilis* for 24 h at 37°C. (E, F) Cells following exposure to *E. coli* for 24 h at 37°C at doses equivalent in cfu to high BT product dose. Experimental details are described in section 2.3.2.

Figure 57. *Bacillus subtilis* and *Escherichia coli* proliferation in the absence and presence of HT29 and Sf9. Growth of *Bacillus subtilis* (▲) or *Escherichia coli* (◆) measured by turbidity at 450 nm in Grace's medium at 27°C (A and B) or DMEM (without phenol red) at 37°C (C and D) after seeding at concentrations equivalent in cfu to 10^{-7} , 10^{-5} and 10^{-3} I.U./cell BT product. Bacteria were seeded into medium without (A and C) or with (B and D) Sf9 or HT29 cells.

Figure 58. Bio-reduction changes in Sf9 and HT29 cells exposed to cell-free culture supernatants derived from growth of *B. subtilis* and *E. coli*. The bacteria were inoculated into Grace's or DMEM at the equivalent of 10^{-3} I.U./cell based on cfu content. Following 8, 12 and 24 h incubation at either 27°C (Grace's) or 37°C (DMEM), cultures were filtered and used for Sf9 and HT29 exposures for the times indicated. Following the different exposures, MTT formazan was measured as described in section 2.4.1. All data points represent the means of three determinations $\pm$ standard deviation.

for 24 h at doses equivalent to BT product. In the presence of gentamicin, no changes in bio-reduction activity were observed, but without antibiotic, formazan production actually increased in proportion to *B. subtilis* dose. Compared to unexposed controls, the increases were 135% for Sf9 and ~140% for HT29. In contrast, *E. coli* exposure gave a 50% decrease at the highest dose which was four-fold more than the highest dose of BT used (10^{-3} I.U. / cell). Similar exposure experiments conducted by immunoblotting indicated that the levels of Sf9 and HT29 actin were reduced during exposure to *B. subtilis*. In the presence and absence of gentamicin, the reductions were about 10% and 20 to 25%, respectively (Figure 54). However, exposures to *E. coli* under similar conditions did not alter actin levels of either cell line and SEM micrographs (Figures 55 and 56) revealed that at the highest dose, no obvious cell loss or damage was visible. Also, *B. subtilis* and *E. coli* cells were seen attached to Sf9 cells, but not to HT29 cells. Additional experiments were carried out to test the ability of these bacteria to proliferate in the presence and absence of animal cells. As shown in Figure 57, bacterial cell production of *B. subtilis* and *E. coli* in Grace's medium without Sf9 cells was appreciable, particularly at the highest dose. However, in the presence of Sf9 cells, the growth of both types of bacteria was dramatically inhibited. Compared to when Sf9 cells were absent, *E. coli* cell production was ~30% at the highest inoculation density, whereas *B. subtilis* cell production was ~1%. In the case of DMEM, neither of the bacteria produced more than 5% biomass regardless of whether or not HT29 cells were present (Figure 57 C, D).

Toxic activity in culture supernatants made from bacterial cultures was also tested (Figure 58). Only cultures derived from *B. subtilis* in Grace's medium were able to cause

morphological damage similar to that observed with *Bt* cultures. The culture supernatants harvested from 12-h and 24-h growths in the absence of animal cells, caused 25% and 65% drops in formazan respectively for *Sf9*. However, in relative terms this toxicity was low because *B. subtilis* cell production at 24 h was 2.15 times higher than that of *Bt* under the same conditions. At short exposure times and low doses, formazan levels of *Sf9* cells increased by ~25%, and although this was also observed in tests with HT29 cells, even longer exposures failed to decrease HT29 formazan levels substantially. In contrast, culture supernatants of *E. coli* did not affect either cell line.

3.9.3 *B. cereus*-like Enterotoxin (BCET)

Recent investigations using commercially available test kits for *B. cereus* diarrhoeal enterotoxin (BCET) indicated that a BCET-like product is associated with BT products and vegetative cells derived from them (Beggs *et al.*, 1996; Damgaard, 1995). The objective here was to determine the potential toxic effects of the BCET standard. As shown in Figure 59, bioreduction was decreased by 85% for *Sf9* and 72% for HT29 when Tecra enterotoxin was used. However, little or no change in bioreduction was observed with similar exposure regimes using the Oxoid BCET standard. The *Sf9* cells showed a transient decrease in bioreduction of ~15% after ~15 min, but by 30 min the formazan production was again comparable to controls. The highest practical dose of Oxoid BCET actually stimulated formazan production of HT29 cells by ~12% between 15 and 60 min. SDS-PAGE/silver staining analysis of the Oxoid standard showed a single 65 kDa polypeptide, whereas the Tecra standard had several polypeptides, which were too dilute to record by photography. SEM analysis following exposure (Figure 60), showed that some *Sf9* cells may have been

Sf9

HT29

CONTROL 500X

CONTROL 3000X

TECRA ENTEROTOXIN
60min - 500X TECRA ENTEROTOXIN
60min - 3000X OXOID ENTEROTOXIN
60min - 500X OXOID ENTEROTOXIN
60min - 3000X

Figure 61. Morphology of HT29 exposed to BCET. HT29 cell monolayers were either exposed to PBS alone (A and B) or BCET standards from Tecra (C and D) and Oxoid (E and F) kits for 60 min in DMEM at 37°C. SEM was carried out as described in section 2.3.2. Micrographs shown here are examples of representative morphologies.

CONTROL 500X

A

CONTROL 3000X

B

TECRA ENTEROTOXIN
60min - 500X

C

TECRA ENTEROTOXIN
60min - 3000X

D

OXOID ENTEROTOXIN
60min - 500X

E

OXOID ENTEROTOXIN
60min - 3000X

F

damaged as indicated by ruptured cell membranes. However, similar changes were not apparent in exposed HT29 cells (Figure 61), but they did form noticeable pseudopod-like formations. Both Sf9 and HT29 cells exposed to Oxoid enterotoxin were essentially normal in shape.

3.9.4 Natural and Recombinant Phosphatidylinositol Phospholipase C (PI-PLC)

Specific assays for measuring PI-PLC activity are complex and not always reliable. Since PI-PLC has been reported to be produced in large amounts by *Bt*, toxicity of *Bt* PI-PLCs derived from commercial sources were used as standards to assess exposure effects in cytotoxicity assays already described in this thesis. Extensive testing using both natural and recombinant enzymes indicated that neither insect nor human cell morphology was altered to any extent. However, at the limit of one unit/mL PI-PLC, a 35 to 40% decrease in bioreduction activity was observed within 10 min of exposure (Figure 62A). Under the same conditions (dose and time), HT29 bioreduction activity was reduced ~30% at five h of exposure. No further changes were seen with longer incubation times. Levels of protein markers, actin and hsp70, were also compared during exposure of MG1 and HT29 cells to PI-PLC. As shown in Figure 62B using actin as the example, no changes in the levels of either marker or cell number were observed over the five-h exposure period.

3.10 Toxicity of Extracellular Products from Vegetative *Btk/Bti* Cultures

3.10.1 Bioreduction and Morphology Assays

From results presented in section 3.9.1, it was established that *Bt* vegetative cells produced the causative agent(s) of animal cell cytotoxicity. Furthermore, *Btk* and *Bti* time-

Figure 62. Bioreduction kinetics and protein content of MG1 and HT29 exposed to PI-PLC.

(A) Cell monolayers were exposed to commercially available, purified *Bt* PI-PLC for the times indicated. Immediately following the exposures, cells were tested for MTT bioreducing ability as described in section 2.4.1. Data points represent the means of three determinations $\pm$ standard deviation. (B) Actin immunoblots of total protein from target cells during exposure to *Bt* PI-PLC and estimates by densitometry of actin levels from attached ($\square$) and shed ($\blacksquare$) cells relative to those of control cells. Also shown is the number of attached ($\circ$) and shed ($\bullet$) cells following exposure.

course expression studies established that this cytotoxic activity was predominantly extracellular and produced mainly within the first 6 h of growth (see related data in Figure 78). Using 6-h *Btk* and *Bti* cultures as sources for this activity, it was found that after filtration to remove residual bacteria, the culture filtrates (CFs) were unaltered in activity. Also, the CF from time points after 6 h caused higher toxicity, but based on vegetative cell content, they were lower than that of the 6-h cultures. The graphs in Figures 63 which compare the MTT bioreduction activity of MG1, *Sf9* and HT29 cells during exposure to dilutions of CF, show dramatic changes in bioreduction activity after only 10 min of treatment. With essentially a 1X CF concentration, an LD₅₀ was obtained in 30 to 60 min. At the lowest CF concentration tested (0.1X) and exposures of only 10 min, both MG1 and HT29 cells exhibited an initial increase in bioreduction activity. In general, HT29 cells were at least ~1.8X more sensitive to low concentrations of CF compared to insect cells. It was further established that the cytotoxicity was quantitatively the same regardless of whether the CF was obtained from *Bt* vegetative cells produced in the presence or absence of animal cells (Figure 63B). Another finding was that essentially equivalent decreases in bioreduction could be obtained if CF from DMEM cultures were used in *Sf9* exposure assays (Figure 63D).

SEM analyses summarized in Figures 64, 65 and 66 indicate that insect and human cells exposed to CF for one h had already showed massive changes in cell morphology which was similar to that observed with either 6 h (human) or 12 h (insect) exposures to whole BT product. At 500X magnification, exposed cells had lost all cell-cell interconnections. At high magnification (3000X), it was apparent that cells had disrupted membranes, with only nuclei appearing to remain intact.

Figure 63. Bioreduction activity of insect and human cells exposed to dilutions of *Btk* culture filtrate. Culture filtrates (CFs) were derived from 5000 × g, 20 min supernatants of six-h growths of *Btk*-BT product (one I.U./μL or 10⁻³ I.U./cell) in either Grace's medium at 27°C or DMEM at 37°C. The CFs were then concentrated to 100 to 1000X and used to expose MG1 (A), *Sf9* (B, D) and HT29 (C) cells at the dilutions indicated. Following treatments, target cells were assayed for MTT bioreduction activity (see section 2.4.1). (B) Shows the bioreduction activity of *Sf9* cells exposed to CF derived from *Btk*-BT product incubated for six h at 27°C in Grace's medium with or without *Sf9* cells. (D) Shows the results of a reciprocal experiment in which *Sf9* cells were exposed to CF derived from *Btk* growth in DMEM for 6 h at 37°C. Data points have been corrected for CF autoreducing activity (see Figure 69C) and represent the means of three determinations ± standard deviation.

Figure 64. Morphology of MG1 cells exposed to culture filtrates. MG1 cell monolayers were exposed for 60 min at 27°C to either PBS alone (A and B) or culture filtrates (CF; Figures C and D) derived from *Btk* grown for six h in Grace's Medium at 27°C. SEM analysis was done as described in section 2.3.2. Micrographs shown are representative of the morphologies observed.

MG1 - SEM

Figure 65. Morphology of Sf9 cells exposed to culture filtrates. *Sf9* cell monolayers were exposed for 60 min at 27°C to either PBS alone (A and B) or culture filtrates (CF; Figures C and D) derived from *Btk* grown for six h in Grace's Medium at 27°C. SEM analysis was done as described in section 2.3.2. Micrographs shown are representative of the morphologies observed.

Sf9 - SEM

Figure 66. Morphology of HT29 cells exposed to culture filtrates. HT29 cell monolayers were exposed for 60 min at 37°C to either PBS alone (A and B) or culture filtrates (CF; Figure C and D) derived from *Btk* grown for six h in Grace's Medium at 27°C. SEM analysis was done as described in section 2.3.2. Micrographs shown are representative of the morphologies observed.

HT29 - SEM

Figure 67. Bio reduction activity of insect and human cells exposed to culture filtrates from *Btk* grown in Grace's medium with and without fetal bovine serum. (A) Foray 48B (100 I.U./20 μ L) was inoculated into each microtitre plate well, which contained 180 μ L Grace's medium either with or without 10% fetal bovine serum (FBS). Growth of *Btk* was monitored by an automated reader at 450 nm. (B, C, D) Comparison of formazan production of HT29 (B), MG1 (C) and *Sy9* (D) exposed to CF derived from *Bt* grown in the presence and absence of FBS. Data points have been corrected for CF autoreducing activity (see Figure 69C) and represent the means of three determinations $\pm$ standard deviation.

Figure 68. Polypeptide profiles of *Btk* culture filtrates. Growth cultures derived from inoculation of medium without FBS with one I.U./ μ L were incubated for the times indicated, filtered and concentrated (40-fold) as described in section 2.1.4. Samples (10 μ L) of each concentrate was separated by SDS-PAGE and stained with either Coomassie (A) or silver (B) stains.

A COOMASSIE STAIN

B SILVER STAIN

Figure 69. Bio-reduction activity of *Sf9* and HT29 exposed to filtrates of culture supernatants harvested during time-course growth of *Btk*-BT product. (A, B) Formazan produced after two h exposure of *Sf9* (A) or HT29 (B) cell monolayers to dilutions of CF harvested from 4, 8, 12, 18 and 24 h incubation of one I.U. Foray 48B / μL of Grace's medium (equivalent to 10^{-3} I.U./cell dose) at 27°C. (C) Formazan produced from dilutions of CF alone (ie. no insect or animal cells present). (D, E) Predicted estimate of formazan produced by *Sf9* (D) or HT29 (E) cells after subtraction of auto-reduction activity (graph C) from data of graph A or B. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 70. Protein yields of culture filtrates and effects on bioreduction activity of *Sf9* and HT29. (A) Extracellular protein yield from *Bt* growth incubation periods. Each cell-free supernatant of culture medium was filtered and concentrated before separation by SDS-PAGE and staining with Coomassie or silver stains. The stained polypeptides were quantified by scanning densitometry and compared with BSA standards of known concentrations. (B) Measurements of formazan production by *Sf9* and HT29 (data from Figures 69 D and E) were plotted as percent maximum cytotoxicity as a function of μg CF protein.

Efforts to examine CF polypeptides by SDS-PAGE were hampered by the presence of fetal bovine serum (FBS), which is used as a supplement in both Grace's medium and DMEM. Therefore, experiments were carried out to determine whether proliferation of *Bt* and toxicity would be substantially affected if FBS was omitted from the target cell growth media. As shown in Figure 67, *Bt* cell accumulation, measured by turbidity during incubation in Grace's with and without 10% FBS, was retarded if FBS was omitted. However, about the same amount of bacterial cells accumulated by eight h without FBS, as six h with FBS. The cytotoxicities of CFs from these 8 h and 6 h cultures, were essentially identical (Figure 67 B, C and D). Also, there were no differences between the cytotoxicities of CFs from Foray 48B and Vectobac. Coomassie and silver stained polypeptides derived from different *Bt* growth times are shown in Figure 68. At zero to six h incubation, staining was minimal and mainly associated with low molecular weight polypeptides (<20 kDa). One polypeptide of 78 to 80 kDa was present at zero h incubation. At eight h, this product was still present, but there were also several others, mainly of low molecular weight (≤ 20 kDa). Also, there were a few other polypeptides which increased in abundance up to the 24 h endpoint. According to the Coomassie stained gels, the major polypeptides were 51, 56, 73 and 125 kDa in size. However, with silver staining of the same samples, the major polypeptides were 45, 78 and 125 kDa. Polypeptides of 67 and 132 kDa which are the most abundant of *Btk*-BT products, were only present in very minor amounts, if at all.

Summaries of results estimating MTT bioreduction of *Sy9* and HT29 cells exposed to dilutions of CF harvested from different times of growth are shown in Figure 69. Without controls, the CF from 12 to 24 h incubations with either Foray 48B or Vectobac, appeared

to stimulate target cell formazan production (Figure 69 A shows exposures with CF from cultures of Foray 48B as an example). However, visual observations by phase contrast microscopy, revealed that the culture wells contained formazan crystalline deposits, which were not associated with either *Sf9* or HT29 cells. Further testing of CF without target cells present, but with MTT, resulted in a dose-dependent accumulation of formazan (Figure 69C), suggesting that a reductase-like activity was produced by *Bt* cells. When the graphs in Figure 69 A and B were corrected for CF autoreduction effects, the data clearly showed a progressive production of cytotoxic activity as vegetative cells were produced over the 24-h incubation period (Figure 69 D and E). The cytotoxic response for HT29 was more rapid than that exhibited by either MG1 or *Sf9* cells. To determine the LD₅₀ for each CF, protein content was quantified (Figure 70A) and data from Figures 69D and 69E were plotted as a function of protein concentration (Figure 70B). These data indicated that the 8 h CF had the greatest toxic activity, yielding LD₅₀ values of ~5 µg/mL for *Sf9* cells and ~1 µg/mL for HT29 cells. Identical results were obtained for CF derived from Vectobac.

3.10.2 Immunodetection of Protein Indicators

Levels of actin and hsp70 were measured during exposures to CF. Generally, data for both insect and human cells were very similar. Data on actin levels of attached and shed cells are shown in Figures 71 A, B and 72 A, B. The time required to give a 50% reduction (half-life) in the level of actin associated with attached MG1 cells was consistent with that of cell number. These values were 1.8 h and 1.4 h, respectively. A similar analysis with HT29 cells gave half-life values of ~one h for actin and ~1.5 h for cell number. Complementary calculations for shed cells could not be made because their actin levels were very low in

Figure 71. Actin and hsp70 immunodetection and cell number of MG1 exposed to *Btk* culture filtrates. Immunoblot assays for actin (A, B) and hsp70 (C, D) were carried out during exposures to 100% CF derived from *Btk* grown for six h in Grace's medium at 27°C. Quantifications of attached cells (○), actin (B) and hsp70 (D), and the predicted number of shed cells (●) are expressed relative to those of control cell levels. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 72. Actin and hsp70 immunodetection and cell number of HT29 exposed to *Btk* culture filtrates. Immunoblot assays for actin (A, B) and hsp70 (C, D) were carried out during exposures to 100% CF derived from *Btk* grown for six h in Grace's medium at 27°C. Quantifications of attached cells (○), actin (B) and hsp70 (D), and the predicted number of shed cells (●) are expressed relative to those of control cell levels. Data points represent the means of three determinations $\pm$ standard deviation.

comparison to the number of cells that were expected to be shed (ie. from the remaining number of attached cells). Similar analyses using hsp70 as a marker is shown in Figures 71 C, D and 72 C, D. At early exposure times (one and two h), hsp70 levels were lower than expected from the number of attached cells. From other experiments, the half-life values were estimated to be ~0.7 h for MG1 and ~0.8 h for HT29. Similar to actin data, the level of hsp70 in shed cells was much lower than expected from cell numbers.

3.10.3 Effects on Animal Cell Protein Synthesis

Since actin and hsp70 levels were often lower than expected following CF exposure, the effects of CF on protein synthesis was investigated using ^{35}S -labelled methionine. As shown in Figure 73, exposure to either Grace's medium alone or 0.1X CF resulted in almost no change in the amount of ^{35}S -labelled polypeptides of HT29. However, when cells were exposed to 0.5X or 1X equivalent of CF, labelling of polypeptides was markedly reduced at 20 to 30 min. At 60 min, the amount of ^{35}S -labelled protein was <20% of that estimated by enumerating attached cell number (or actin) in Figure 72. During exposure to either 0.5X or 1X CF, only one additional ^{35}S -polypeptide was seen in relation to control cells treated with either Grace's medium alone or 0.1X CF. This 40 to 45 kDa polypeptide was seen at 30 and 60 min exposure to 0.5X CF, and 20 and 30 min exposure to 1X CF, but was not present at later incubation time intervals.

By shifting the ^{35}S -methionine labelling interval, it was possible to monitor in more detail the ability of CF-exposed cells to carry out protein synthesis. With 30 min intervals for incorporation of ^{35}S -methionine, polypeptide synthesis in attached HT29 cells was rapidly reduced by >90% soon after CF exposure (Figure 74). The half-life of protein synthesis for

Figure 73. ^{35}S -methionine-labelled polypeptides of HT29 incorporated prior to culture filtrate exposure. HT29 cells were exposed to ^{35}S -methionine for 30 min and then exposed for various lengths of time to fresh Grace's Medium alone (not shown), and fresh Grace's Medium adjusted to 0.1X (A), 0.5X (B) or 1X (C) of 6-h *Bt* culture filtrate (CF) using a 1000X stock. Graph D shows a semi-quantitative estimate of the results by densitometry of total labelled polypeptides relative to those of control cells.

Figure 74. ^{35}S -methionine-labelled polypeptides of HT29 incorporated during culture filtrate exposure. HT29 cell polypeptides were incubated with ^{35}S -methionine for 30 min pulse intervals before and during exposure to 0.5X *Btk* CF (indicated by horizontal bars in Graph D). Polypeptides from cells that were attached (A and B) and shed (C) during CF exposure were separated by SDS-PAGE and label was detected by autoradiography for six h (A) and 24 h (B and C). Culture filtrates were derived from *Btk* grown for six h in Grace's Medium at 27°C.

Figure 75. ^{35}S -methionine-labelled polypeptides of *Sf9* incorporated during culture filtrate exposure. *Sf9* cell polypeptides were incubated with ^{35}S -methionine for 30 min pulse intervals before and during exposure to 0.5X *Btk* CF (indicated by horizontal bars in Graph C). Polypeptides from cells that were attached (A) and shed (B) during CF exposure were separated by SDS-PAGE and ^{35}S label was detected by autoradiography for six h (see section 2.5.3 for details). Culture filtrates were derived from *Btk* grown for six h in Grace's Medium at 27°C.

0.5X CF exposure was only 15 min which is about half that computed using other indicators. Analysis of the shed cell fraction further indicated that the amount of ^{35}S -label was only about 7% of the control. Attached cell ^{35}S -label was lower after only 10 min exposure, and after the next pulse interval it was reduced to background levels. Similar experiments were conducted with *Sf9* cells. The data presented in Figure 75 agree with the HT29 data, indicating a half-life of ~15 min. The *Sf9* cells that were shed during exposure to CF had little or no associated ^{35}S -label ($\leq 1\%$ of control). A re-examination to track the appearance of the 40 to 45 kDa polypeptide observed in the pre-labelling experiments was negative, suggesting that the polypeptide was a degradation product and not *de novo* synthesized. The combined results indicated that a major CF toxic effect is on the target cell protein synthesis machinery.

3.10.4 Binding Assays with ^{35}S Sulfur-labelled Polypeptides of *Btk*

The possibility that some component(s) of the CF actually binds to target cells thereby causing their lysis was investigated. The strategy used for these assays is described in Figure 76A. The results from the autoradiograph showing the distribution ^{35}S -methionine labelled *Btk* cells (pellet fraction) and extracellular proteins (S) in the CF are presented in Figure 76B. The HT29 and *Sf9* cells that remain attached after CF exposure appeared to be associated with several CF ^{35}S -polypeptides, the major one being 30 to 32 kDa in size and minor ones measuring about 15, 22, 40, 45 and 80 kDa. In a second experiment, this assay was modified in that the target cell exposure time was limited to a 30 min time-course; CF and *Btk* cells (pellet) were derived from growth at 27°C and 37°C and shed target cells were collected by centrifugation and extensive rinsing prior to protein solubilization and electrophoresis. These experiments showed that maximum binding of CF or *Btk* polypeptides to either *Sf9* or HT29

Figure 76. Binding of ^{35}S -methionine-labelled *Btk* polypeptides to *Sf9* and HT29. (A) Method outline: Foray 48B product (one I.U. / μL Grace's medium without FBS) was incubated for six h in medium containing methionine and then for 18 h in medium containing ^{35}S -methionine. The *Btk* culture was then separated into cell pellet (P) and cell-free supernatant (S) fractions. The pellet was resuspended in an identical volume to the supernatant and both were used to expose *Sf9* and HT29 monolayers. Following exposure for one h, the shed cells (SC) were removed and those remaining attached (AC), were rinsed with fresh Grace's medium (FBS-minus) and both SC and AC were solubilized with Laemmli buffer. (B) Polypeptides from these cells were analysed by SDS-PAGE and autoradiography as described in section 2.5.3.

B PAGE / AUTORADIOGRAPHY 48 h, -80 °C

target cells occurred within 5 min of exposure. No additional polypeptides bound to target cells if CF was derived from 37°C cultures, even though the distribution and abundance of labelled polypeptides were obviously different at the two incubation temperatures. When cells were exposed to labelled polypeptides from cultures that were pelleted, the major interactive polypeptide was again 30 to 32 kDa.

3.11 Characterization of the Cytotoxic Component(s) of Culture Filtrate

3.11.1 Properties

To obtain information about the properties of the cytotoxic component(s), CFs were pre-treated in several ways before conducting exposure bioassays (Figure 77). Passage of the CF through various sized molecular weight cut-off filters resulted in almost all of the cytotoxicity remaining in the ≥ 30 kDa hold-up fraction, and $<2\%$ was detected in the ≥ 100 kDa flow through (Figure 77A). Pre-treatment of the CF for $\leq$ six h with temperatures between -20°C to 50°C resulted in little or no loss in activity, but treatments for ≥ 1 min between 60 to 100°C resulted in inactivation (Figure 77B and C). Pre-incubation of the CF for ≤ 2 h at 27°C or 37°C with either trypsin (0.025 to 0.25%), protease inhibitors (PMSF or TLCK), EDTA (0.1 to 10 mM), NAD (≤ 0.1 mM) or ATP (≤ 0.1 mM) had no effect on cytotoxicity (Figure 77D). However, pre-incubation of the CF for 30 min at 37°C with a broad-spectrum protease such as proteinase K, resulted in a 77% loss of the cytotoxic activity in bioassays when 100 $\mu\text{g/mL}$ proteinase K was used for 90 to 120 min. In the same period, proteinase K in Grace's medium alone caused $<5\%$ cytotoxicity. Attempts were made to differentially precipitate the toxic component(s) with 80% ethanol or 5% trichloroacetic acid

Figure 77. Effects of various treatments on the cytotoxic activity of culture filtrates. To obtain data shown in A to F, eight h CF from Grace's medium without FBS was pre-treated in the following ways: (A) passage through molecular weight exclusion filters; (B) preincubation at various temperatures and times followed by cooling to 27°C and exposure of *Sf9* cells for 2 h at 27°C to derive half-lives (C) at the different temperatures; (D) incubation with various agents for 30 min; (E) adjustment of pH with sodium citrate followed by sedimentation of insoluble material; and (F) precipitation or "salting out" with ammonium sulphate. Following treatments, cytotoxicity was monitored in 2-h bioassays using *Sf9* cells at 27°C. Data points are the means of three determinations $\pm$ standard deviation. Very similar results to those shown here were obtained with HT29 bioassays and also with CF derived from Vectobac.

(TCA), but no activity was recovered from either the supernatant or pellet fractions after dialysis. When CF proteins were precipitated by adjusting pH using citric acid as described by Warren *et al.* (1996), toxicity could be detected in either the soluble or insoluble fractions (Figure 77E), but SDS-PAGE analysis showed that no enrichment of any CF proteins was obtained. Partial enrichment of the toxic substance(s) was achieved by “salting out” using ammonium sulfate (Figure 77F). At 30% ammonium sulfate saturation, about 30% of the total protein material was precipitated, but the toxic activity remained almost totally in solution. Following centrifugation, the supernatant was adjusted with more ammonium sulfate to give 70% saturation (final). This resulted in precipitation of the activity. By comparing dilutions of this enrichment (following dialysis) with that of CF, it was determined that about 90% of the total activity was recovered and the specific activity was increased by ~2.3X. From PAGE analysis, no specific proteins were selectively precipitated during ammonium sulphate addition.

3.11.2 Expression and Subfractionation of the Cytotoxic Substance(s)

The study of toxicity expression by *Bt* summarized in Figure 78 shows that the highest specific activity of CF cytotoxicity was obtained from cultures grown for 6 to 8 h. Expression of cytotoxicity was consistent with the number of vegetative cells, but not with spore levels (Figures 78C and D). At the early stages of vegetative cell production (ie. 8 h) the amount of extracellular protein produced was <15 µg/mL, but the toxic activity was >95% of that observed with CF from later growth times, such as 24 h. However, to obtain some information about CF homogeneity 8, 12 and 24 h CFs were fractionated by exclusion column chromatography. These experiments are summarized in Figures 79 to 84. As shown in

Figure 78. Expression of cytotoxic activity in culture filtrates during *Btk* proliferation. (A) Foray 48B was inoculated at a concentration of one I.U./ μ L (equivalent to the 10^{-3} I.U./cell dose) into Grace's medium without gentamicin. The number of vegetative cells, spores and the protein content of the culture filtrate was quantified after incubation for various time intervals. (B) The specific activity data for both Sf9 and HT29 cells ($\circ$ and $\bullet$, respectively) were derived from results given in Figure 70. (C and D) Show comparisons of specific activity (cytotoxicity/ μ g of CF) to the number of spores and vegetative cells present, respectively. Data points represent the means of three determinations $\pm$ standard deviation.

Figure 79. Relative protein content and *Sf9* bio reduction activity from fractions collected by size exclusion chromatography. Culture filtrates were derived from *Btk* grown for either 8, 12 or 24 h at 27°C in Grace's medium. Before loading on Sephadex G-100 columns, the CFs were concentrated by 1000X. Further experimental details are described in section 2.1.5. Cytotoxicity of each filtrate fraction was determined by *Sf9* MTT bio reduction activity following a two h exposure at 27°C. Results are expressed either as a percent of maximum toxicity (A, B and C) or as specific activity (% cytotoxicity / O.D.₂₈₀) (D, E and F). Pooled fractions are indicated with square brackets.

Figure 80. Relative protein content and HT29 bioreduction activity from fractions collected by size exclusion chromatography. Culture filtrates were derived from *Btk* grown for either 8, 12 or 24 h at 27°C in Grace's medium. Before loading on Sephadex G-100 columns, the CFs were concentrated by 1000X. Further experimental details are described in section 2.1.5. Cytotoxicities of each filtrate fraction following a two h exposure at 37°C were determined by HT29 MTT bioreduction activity and expressed either as a percent of maximum toxicity (A, B and C) or as specific activity (% cytotoxicity / O.D.₂₈₀) (D, E and F). Pooled fractions are indicated with square brackets.

Figure 81. Polypeptide profiles of culture filtrate fractions collected by size exclusion Chromatography. A, B and C gels correspond to 8, 12 and 24 h CF fractions from Sephadex G-100 columns (see Figures 79 and 80). The last three lanes of each set show proteins of pooled fractions (indicated with numbers above the lanes and shown with square parentheses in Figures 79 and 80) and total CF (TOT). The first lane of every gel has the molecular weight standard (M). From top to bottom these are the following in size: 126, 80, 48, 28 and 19 kDa.

A

M 6 7 8 9 10 11 12 13 14

M 15 16 17 18 19 20 7-14 15-20 TOT

B

M 6 7 8 9 10 11 12 13 14

M 15 16 17 18 19 20 8-11 12-18 TOT

C

M 6 7 8 9 10 11 12 13 14

M 15 16 17 18 19 20 7-12 15-21 TOT

Figure 82. Relative protein content and *Sf9* bioreduction activity from fractions collected by anion exchange chromatography. Pooled CF derived from *Btk* grown for 24 h at 27°C in Grace's medium were concentrated by 1000X, and fractionated using a miniature sephadex DEAE-50 column as described in section 2.1.5. Approximate protein concentration was determined by measuring the O.D. at 280 nm and cytotoxicity of each fraction was determined by *Sf9* MTT bioreduction assay after a two h exposure at 27°C. Cytotoxicity was expressed either as a percent of maximum toxicity (A) or as a specific activity (% cytotoxicity / O.D.₂₈₀) (C). Pooled fractions are indicated with square brackets. (B) Example of microwells showing the MTT formazan produced by *Sf9* cells exposed to CF fractions.

Figure 83. Relative protein content and HT29 bioreduction activity from fractions collected by anion exchange chromatography. Pooled CF derived from *Btk* for 24 h at 27°C in Grace's medium were concentrated by 1000X, and fractionated using a miniature Sephadex DEAE-50 column as described in section 2.1.5. Approximate protein concentration was determined by measuring the O.D. at 280nm and cytotoxicity of each fraction was determined by HT29 MTT bioreduction assay after a two h exposure at 37°C. Cytotoxicity was expressed either as a percent of maximum toxicity (A) or as a specific activity (% cytotoxicity / O.D.₂₈₀) (C). Pooled fractions are indicated with square brackets. (B) Example of microwells showing the MTT formazan produced by HT29 cells exposed to CF fractions.

Figure 84. Polypeptide profiles of culture filtrate fractions collected by anion exchange chromatography. DEAE-50 column fractions of CF derived from 24 h *Btk* growth that contained measurable protein by optical density (Figures 82 and 83), were separated by SDS-PAGE. Polypeptides were silver stained. Lane numbers correspond to column fractions, pooled fractions or total CF (TOT). The first lane of every gel shows the molecular weight standards used (M). From top to bottom these are the following in size: 126, 80, 48, 28 and 19 kDa.

A **B**

Figure 85. Phosphorylation of polypeptides in culture filtrates using ^{32}P -nicotinimide adenine dinucleotide (^{32}P -NAD) as donor. Concentrated (1000X) CF derived from *Btk* grown for 24 h in Grace's medium at 27°C was incubated with ^{32}P -NAD as described in section 2.1.4. Examples of assays are shown in (A). Coomassie stain of SDS-PAGE gels of total protein is on the left side and autoradiography of ^{32}P -incorporation is on the right. (B) Quantification of the ^{32}P incorporated into the 45 kDa protein. The dotted lines show results from three separate experiments and the solid line corresponds to the average of these data points $\pm$ standard deviation.

Figure 79, when size exclusion columns were used (Sephadex G-100), CF derived from eight h *Bt* growth gave one major peak of activity, while CFs from 12 and 24 h growth exhibited progressive broadening of the peak. When these results were replotted to give a specific activity (Figures 79 D, E and F), the highest toxicity levels were located in four fractions of the eight h CF, whereas the other CF's had activity spread over 10 or more fractions. Tests were done to determine if the fractions encompassing various peaks of activity would be synergistic, but no additive effects were obtained. A similar analysis was conducted with the same fractions, but using HT29 cells. Results in Figure 80 show that fractions cytotoxic to *Sf9* were also active against HT29. High specific activity of the eight h CF was limited to one major and one minor peak, and those of 12 and 24 h were similar but spread over more fractions. The latter 12 and 24 h profiles could be considered to be made up of two peaks of similar portions as those of the 8-h CF. Silver stained polypeptides that were present in individual and pooled fractions are shown in Figure 81. In each case, there were many different sized polypeptides in fractions with the highest toxicity. These fractions were impoverished in polypeptides >90 kDa.

As an alternate fractionation strategy for analysis of the cytotoxic substance(s) in CF, an anion exchange chromatography method was used. For this purpose, only CF from 24 h *Bt* cultures was used with the expectation of obtaining a separation of factor(s) based on size and charge. After collection of each fraction, protein content and cytotoxicity was determined. In measuring cytotoxicity of these fractions to *Sf9* cells, there were four peaks of high toxicity and one of low toxicity (Figure 82). When HT29 cells were exposed to these same fractions, the specific activity pattern was close to that observed for *Sf9* (Figure 83).

The highest activity to both cell lines was obtained from fractions 24 and 29. An analysis of the polypeptides present in each fraction is shown in Figure 84. These gels showed that a fractionation was facilitated, but this fractionation was still too crude to resolve the toxic components into a single polypeptide, if this is the case.

3.11.3 ^{32}P -NAD and ^{32}P -ATP Phosphorylation Assays

Since some bacterial toxins use cofactors, in their activity such as nicotinamide adenine dinucleotide (NAD) or adenosine triphosphate (ATP), experiments were done to determine if any proteins in the CF act as receptors for NAD or ATP. For NAD assays, radiolabelled NAD (^{32}P -adenylate) was incubated with CF for various periods of time at 27°C or 37°C and then ^{32}P -labelled polypeptides were detected with SDS-PAGE and autoradiography. As shown in Figure 85, at least one product (~45 kDa) was detected. However, prolonged incubations could result in a loss of this phosphorylation by ~80 min as indicated in Figure 85B. Similar assays using $\gamma^{32}\text{P}$ -ATP gave no labelled products. Assays conducted with Foray 48B PIB and Vectobac PIB (see section 3.8.2) showed that when unlabelled NAD or ATP was added to the exposure system or used in pre-incubations, no change in toxicity was observed (see section 3.11.1). The pooled fractions of high toxicity obtained from anion exchange chromatography columns, were also tested for ^{32}P -NAD binding ability. Only the last pool (fractions 26 to 30) showed the 45 kDa labelled polypeptide.

4. DISCUSSION

4.1 Physical and Biological Characterization of Commercial BT Products

At the time that this thesis was initiated, there was little published information on BT product contents. Product labels and information sheets from manufacturers (Material Safety Data Sheets), and regulatory information on pesticide products published by Agriculture and Agri-Food Canada in 1996, indicated that they contain “active ingredient” described as *B. thuringiensis* subspecies *kurstaki* or *israelensis*, without much further clarification. The potency (International Unit) given for the different commercial BT products (see Table 2) is not a very meaningful or precise descriptor for environmental health assessment purposes because it is only an extrapolated, relative measure of insect lethality (Beegle and Yamamoto, 1992).

At present, publications critically describing BT product contents, have come largely from work done in the last few years, and include methods to quantify such products from sampling of field applications (Beggs *et al.*, 1996; Rancourt *et al.*, 1996; Seligy *et al.*, 1997a; Seligy *et al.*, 1997b; Tayabali and Seligy, 1995; Tayabali *et al.*, 1996). According to microscopical analysis (phase contrast and scanning electron microscopy), BT products are remarkably similar to each other, but they are also very complex as shown in Figures 3, 4 and 5. These data in conjunction with dilution and/or treatment with a broad-spectrum protease (proteinase K), demonstrate that BT products consist of a liquid as well as a semi-solid phase comprised of amorphous particles, crystalloid parasporal inclusion bodies (PIBs) and spores. The liquid fraction and amorphous particles contain proteinaceous material, carbohydrates and extracellular nucleic acids, and additives such as growth inhibitors, foliage stickers (eg.

oils) and tracer dyes (Seligy *et al.*, 1997b). The intact PIB structures of whole BT products are variable in number and appear as bypyramidal (*Btk*) or spherical (*Bti*) crystalloids (Figures 3 and 4). When semi-purified by sucrose gradient ultracentrifugation (Figure 44 and 45), they matched PIB structures described for laboratory strains of *Btk* and *Bti* after culture in liquid media (≥ 72 h) (Chilcott and Wigley, 1994; Solontsov and Yudina, 1996). Procedures to purify intact PIB structures and intact pro- δ -endotoxins from other BT product constituents are non-trivial. They are in fact complex, time-consuming and inefficient (Table 6). The use of recombinant DNA methods has greatly assisted in the endeavour to obtain pure δ -endotoxins variants (Masson *et al.*, 1989). To ensure that an enrichment of PIB is achieved, microscopical examination is very much required as even after three cycles of so-called purification, most PIB-enriched fractions were still contaminated with variable amounts of spores. In our experience, the spores can only be eliminated by protease solubilization of the PIBs, followed by differential centrifugation and filter-sterilization of the supernatant using 0.22 to 0.45 μm pore size membranes. Alternatively, an antibiotic must be added to inhibit spores and other possible microbial contaminants. A critical review of existing literature suggests that few if any PIB purification strategies have actually used similar precautions to prevent spore effects.

Critical enumeration of spore content of BT products carried out by our laboratory, including myself, substantiate that spore numbers are very high ($\sim 10^{10}$ cfu/mL) and extremely stable to heat, proteases, detergents and pH extremes (0.8 to 14) (Seligy *et al.*, 1997a). The spore content of *Btk*-BT products is actually very consistent with claims of I.U. content (Table 2) which is contrary to earlier claims that there was no connection between spore

other proteins associated with the crude preparations of PIBs.

For *Bti*-based Vectobac product, the sizes of the major abundant polypeptides also agree with reports describing *Bti* PIB proteins (Thomas and Ellar, 1983; Cheung and Hammock, 1985). The 78 kDa and 134 kDa polypeptides would correspond respectively to the activated (truncated) and the pro- δ -endotoxin (precursor) of CryIV. The 27 kDa moiety is probably the cytolytic toxin (CytA), which is reported to lyse both insect and mammalian cells (Wu and Chang, 1985). According to staining intensity of polypeptides after PAGE, the *Btk* precursor and truncated δ -endotoxins represent approximately 49% and 20% of the total protein of BT products, whereas the Cyt A toxin in *Bti*-BT product was the most abundant polypeptide, and therefore a concern for toxicity to human cells. Following product treatment by centrifugation, it was revealed that at least 99% of the protein content was found in the particulate phase (Figure 5). The peptides in the liquid phase (<1% of total), when concentrated 10-fold, measured 17, 20, 25, 35 and 75 kDa in size and were therefore not in the size range of activated or precursor δ -endotoxins (Figure 42A). Treatment of the particulate phase with a broad-spectrum protease such as proteinase K over a period of 12 h, resulted in a 100% loss of PIB structures and polypeptides, but $\leq 10\%$ loss in spores, confirming that the precursor and activated δ -endotoxins (also the *Bti* cytolytic toxin) were extra-spore associated.

4.2 Development of *in vitro* Assays to Assess BT Product Toxicity

Guidelines for simulating field doses in the *in vitro* bioassays conducted here were derived from data on BT products summarized in Table 2, Figure 4, and aircraft field

applications (Seligy *et al.*, 1997b). The high dose used in this thesis (3 I.U./mm² or 10⁻³ I.U. per animal cell) corresponds to an application dose of 30 B.I.U./hectare, which is routinely used in aerial spray operations (Seligy *et al.*, 1997b). The actual amount ingested by a target larva may vary considerably from 0.5 to 27 I.U./larva (van Frankenhuyzen and Payne, 1993). Since doses <0.5 I.U./larva may only cause cessation of feeding, concentrations as low as 10⁻⁷ I.U. per animal cell would have been appropriate to study. In fact, doses as low as 10⁻⁹ I.U./animal cell were also examined.

Currently, *in vivo* bioassays comprised of whole insect larvae are used to establish BT product potency (toxicity), but these assays are fairly labour-intensive and time consuming, requiring about 4 to 7 days (Beegle and Yamamoto, 1992). The trend towards use of *in vitro* cellular assays is partly to avoid problems of insect rearing, the constant maintenance of a specialized ecosystem for generating the required larval-stages. Also, *in vivo* bioassays are difficult to standardize because of uncertainty of dose acquisition and injury due to handling. *In vitro* assays are potentially less limited in throughput and they provide the opportunity to make side-by-side comparisons of non-target organism cell effects, such as human.

When this thesis was initiated, an extensive review of the literature indicated that there were only a few *in vitro* assays for assessing δ -endotoxin toxicity (see review section 1.6.2). Drawbacks to these assays are the limited choice of toxicity endpoints and lack of automation. Overall, microscopical evaluation of cell morphology during exposure probably is the most revealing and low-cost way to monitor toxic effects. However, by itself this approach is not very practical for objective quantification of large numbers of samples. Microscopy in conjunction with dyes such as trypan blue uptake by non-viable cells has been one strategy

used for assessing δ -endotoxin toxicity (Gill and Hornung, 1987; Smith *et al.*, 1996; Thomas and Ellar, 1983; Vachon *et al.*, 1995). This type of assay was used early to test BT products in this thesis, but it was found to involve too many steps and was inaccurate in estimating non-viable cells that had shed during the assay. Also, trypan blue does not stain *Bt* vegetative cells so they are not easily noticed. As a consequence, one objective of this thesis was to develop an adaptable, multi-indicator assay that could be used to monitor both multi-component toxins and even cell defence responses. The following subsections discuss some important observations leading to the optimization of the various assays developed and used here.

4.2.1 Bioreduction/Cell Viability Assays

One of the most successful bioassays for determining mammalian cell viability and enumeration employs yellow tetrazolium-based dyes. These dyes are reduced to indigo to red products by cell-mediated electron transport mechanisms and therefore the amount of reduced dye can measure indirectly cell doubling and metabolic damage during exposure to xenobiotics and radiation (Barile *et al.*, 1993; Borenfreund *et al.*, 1988; Carmichael *et al.*, 1987; Hsieh and Acosta, 1991; Morgan *et al.*, 1991; Mosmann, 1983; Roehm *et al.*, 1991; Ryghseter *et al.*, 1992; Weichert *et al.*, 1991). For the work presented here, both MTT and XTT-based assays were adapted to various sized multiwells and semi-automated procedures to enable multi-step treatments and also replication. A good part of the early thesis work was focussed on optimizing the use of these dyes, and configuring them for analysis of BT products, and insect and human cells (Tayabali and Seligy, 1995). Part of the optimization involved selecting useful indicator concentrations. For MTT, it was found that above 2.4 mM

and 2 h of incubation, formazan levels dropped as a consequence of cell detachment caused by intracellular formation of large, insoluble formazan crystals. This effect could be eliminated by using 1.2 mM MTT for two h, which turns out to be 50% shorter than assays earlier described for mammalian cell assays (Mosmann, 1983; Carmichael *et al.*, 1987). Under these conditions cell number was found to be directly proportional to the amount of formazan produced by either insect or human cells. The main differences between insect and human cell lines are cell size (human cells are ~30% larger than insect cells), assay temperature (37°C versus 27°C), and glucose content in culture media which may influence formazan production (Vistica *et al.*, 1991). It was found that the formazan produced by MG1 and Sf9 cells cultured at 37°C was about 30% higher than that at 27°C. Most of the other human cells examined were comparable in bioreduction activity to insect cells grown at 37°C. However, HT29 cells produced about 9X more formazan than insect cells. This output may be related to HT29 mitochondrial activity coupled to differentiation (Lu *et al.*, 1992).

When the MTT-based assay was applied to BT product exposures (section 4.3.1), it was found that vegetative *Bt* cells tend to bind to animal cells and trap along well edges (Figures 15, 16, 17, 23, 27, 29, 30). Since this binding could add to the overall formazan production, the MTT bioreduction assay was modified by using washes and larger wells. The formazan produced by vegetative *Bt* cells has been estimated to be about 50-fold higher than that of spores (Seligy *et al.*, 1997a). Most of this formazan remains bound to spores and vegetative cells, but some extracellular MTT-reducing activity is also produced (Figure 69C). This MTT-reducing activity may be secreted or shed as membranous debris during the later stages of culture. The bioreductive capacity of large numbers of vegetative *Bt* cells at 24-h

endpoints could be a serious background problem. It was also a problem when using the XTT dye (Figure 10), since the diffusible, water-soluble formazan could not be removed with vegetative cells. However, the XTT-based assays proved very useful in assays involving cell-free extracts and automated kinetic studies conducted over the span of 24 h.

Investigations into the bioreduction of MTT and XTT using metabolic uncouplers and inhibitors such as 2,4-dinitrophenol and sodium azide which act on the mitochondrial electron transport system, showed that they only partly impaired bioreduction activity to about ~40% of control cells (Figure 11 A and B; see also Berridge *et al.*, 1996; Tayabali and Seligy, 1995). This decrease in bioreduction activity was not accompanied by cell detachment or lysis and may therefore only involve a few of the pathways for dye reduction. This view is supported by other experiments (not presented in this thesis) similar to those of Berridge and colleagues (1996) which demonstrated that MTT and XTT were reduced in both isolated mitochondrial and microsomal fractions. These results are in striking contrast to those using the ionophore A23187 in which both increases and decreases in formazan production were seen. In a 4-h exposure of Sf9 and HT29 cells, there was a dramatic increase in formazan production (~130 to 155%) at doses of 0.5 to 5 μ M. However, at doses ≥ 25 μ M and exposures spanning 24 h, formazan activity actually decreased by 73 to 85%. The early work of Pressman (1976) suggested that A23187 caused the formation of calcium-selective channels in plasma membranes. If this is true, the early stimulation of formazan production may have effected an increase in MTT uptake. Alternatively, calcium influx from ionophore effects could have stimulated activity of MTT-reducing enzymes. Monette and colleagues (1997) have used A23187 to increase calcium uptake by Sf9 cells in order to investigate

to facilitate interspecies comparisons, and also to standardize assays for others to use, only commercially-produced antibodies were investigated.

In the summary presented in Table 4, most immunoprobes were found to be either weakly detected at dilutions >10 or unreactive according to immunoblotting criteria using proteins from insect and human cells. Of the 14 immunoprobes tested, nine were potentially useful for detection of mammalian proteins, and only two of these (anti-actin and anti-hsp70) were effective in detecting both insect and human cell antigens. These probes were useful because they were specific for only the target eukaryotic proteins, and not those of prokaryotic cells, making it possible to assay without interference of *Bt* cells, unlike the XTT bioindicator assay. Of the two, the actin probe was empirically the most reliable for verifying attached cell number. Another important feature of this probe albeit not exploited here, rests on the claim that this antibody probe detects all known actin types such as muscle and cytoplasmic isoforms, differentiated according to isoelectric point (Lessard, 1988).

The antibody probe for the heat shock 70 proteins exploits highly conserved epitopes common to insect and human cell hsp70 (Anderson *et al.*, 1993). This monoclonal antibody detects a 73 kDa protein thought to be constitutively produced, as well as a 72 kDa protein which has been reported to be inducible by different cell stressing stimuli (Craig *et al.*, 1993). Using this immunoprobe, both 72 and 73 kDa products were detected in control MG1 and *Sf9* cells (Figures 20 and 21). The co-expression of 72 and 73kDa proteins has been observed with untreated *Sf9* cells (Huang *et al.*, 1994) as well as several human cell lines (Anderson *et al.*, 1993). However, only one product measuring 73 kDa was observed with the human cells tested (Figure 31). The production of the hsp70 proteins in control cells may be

necessary to meet the increased demands of proliferation and passage-related functions such as protein synthesis, nascent peptide folding, oligomeric assembly and membrane translocation (Beckmann *et al.*, 1990; Craig *et al.*, 1993; Stuart *et al.*, 1994). Additional tests to determine if *Btk* produced cross-reactive proteins showed that there were none expressed before or after temperature elevation (35 to 60°C), such as reported previously by Shakoori and colleagues (1991, 1992).

4.3 Analysis of Cytotoxic Effects Induced by Whole BT Products

4.3.1 24-h Exposures Assays

In the initial objective to assess BT product effects on different cell types, exposures were conducted over a wide range of doses with intermediate and pre-set endpoints such as 24 h. As a tool to discriminate effects due to spores and other ingredients, an antibiotic (gentamicin) was also used to block spore germination. This antibiotic is commonly used in cell culture laboratories and had been used to treat an ocular infection following exposure to the *Btk*-based product, Dipel (Samples and Buettner, 1983). Our recent studies with several different antibiotics, have shown that the concentration of gentamicin used in animal cell exposure experiments described in this thesis (50 µg/mL), was an effective spore and vegetative cell biocide (Rancourt *et al.*, 1996). With this control, only moderate changes were seen in bioreduction activity (Figures 12 and 22), morphology (Figures 13 and 23), actin and hsp70 levels (Figures 20 and 21) and cell shedding and lysis (Figures 18 and 24), in comparison to culture results in the absence of antibiotic.

These combined results using whole BT products agree with those of Ignoffo and

colleagues (1977), who demonstrated that if cabbage looper larvae were fed spores and PIB crystalloids concurrently, lethality was highest (~200-fold) when such treatments did not include antibiotic. The seriousness of spore contamination effects on assay results using so-called purified δ -endotoxins (Fast, 1977) has been pointed out in reassessment studies (van Frankenhuyzen, 1994). Through the use of gentamicin, it was possible to trace the cytopathology of BT products to extracellular proteins of vegetative cells which were generated from BT products (spores). Based on these types of studies, 24-h exposure assays were excessive and did not truly discriminate between dose levels. The threshold where an “all-or-nothing” response was detected ($\sim 10^{-9}$ I.U./cell) corresponds to a dose of about one spore per well. In toxicological terms this would be the “No Adverse Observed Effect Level” (NAOEL). At 10^{-9} I.U./cell a positive response is essentially due to a single spore that would eventually produce an excess of 10^{10} vegetative cells in the latter half of a 24-h exposure (Seligy *et al*, 1997a).

Another important observation from the 24-h exposure assays was that in some cases, doses close to the NAOEL caused a 110% to 120% increase in formazan activity similar to that of the A23187 ionophore experiments. However, the possibility that presence of a single PIB crystalloid caused this increase by cell membrane damage was ruled out with detailed experiments involving gentamicin and enriched PIB samples (see section 4.7.1). The activity of dehydrogenases (MTT-reducing enzymes), which are critical for glycolysis and the tricarboxylic acid cycle, may increase as ATP demand increases. At 24-h exposures with doses above the NAOEL ($\geq 10^{-7}$ I.U./cell), formazan production by target cells (visualized by microscopy) was always essentially zero. All the formazan measured by spectrophotometry

could be accounted for by the presence of formazan associated with vegetative *Bt* cells. At BT doses $>10^{-3}$ I.U./cell, formazan levels increased due to bound vegetative cells as well as presence of MTT-reducing enzymes released into the growth medium by vegetative cells. This was shown by assaying cell-free supernatants (or filtrates) of *Bt* vegetative cells with and without target cells present. Further evidence supporting this conclusion is found in experiments of Figure 69C, which demonstrate that *Bt* supernatants (filtrates), derived from 12 to 24-h growth cultures, produced large amounts of extracellular formazan which adhere to the surfaces of animal cell monolayers.

Microscopical observations along with replating experiments confirmed the non-viability of the remaining, attached animal cells after BT product exposure. At higher morphology resolution (SEM), these cells were disrupted (Figures 13 and 23). Another study has shown similar changes in gross cytopathology, including excessive vacuolarization and electron-lucid cytoplasm, when either TN-368 (*Trichoplusia ni*) or FPMI-CF1 (*Choristoneura fumiferana*) cells were exposed *in vitro* to 50 to 5000 $\mu\text{g/mL}$ of solubilized and activated *Bt* δ -endotoxin (Johnson, 1981; Murphy *et al.*, 1976; Nishiitsutsuji-Uwo and Endo, 1979). In these experiments, it is unlikely that these researchers had taken precautions to prevent and monitor effects due to spore contamination. Cell damage of this type has also been described in lysis of midgut cells of *Manduca sexta* (silkworm larva) after 2.5 h exposure to 0.5 μg of PIB preparation per larva (Spies and Spence, 1985). In these experiments, the toxicant was pre-treated with ultraviolet C (UVC) light for 15 min to inactivate spores, but spore survival was not actually assessed following irradiation. Efforts by us to use this same UVC strategy on BT products (spores of *Btk* and *Bti*) were only partially successful

larva treated with *Bt* subsp. *thuringiensis* spores, which germinated in the midgut (Sutter and Raun, 1967). These extrusions of cytoplasm may represent the initial stages of a general pathway of cell fragmentation, leading to death (Kumar *et al.*, 1992), and could result from disruptions of actin microfilaments (Percy and Fast, 1983). However, the combined results presented in this thesis, indicate that all marker proteins decreased upon exposure to commercial BT products (Figures 20, 21, 31, 32, 33) and that the rate of loss of total protein was about the same and consistent with loss of attached cells. Two possible exceptions to the general cytoplasmic protein damage observations were: hsp72 of insect cells and cyclin of HT29 cells. The levels of hsp72 in MG1 and S9 were rapidly lowered compared to hsp73 or actin levels. Since it has been suggested that most, if not all proteins transiently interact with hsp70 proteins (Beckmann *et al.*, 1990), early losses of hsp72 may interfere with translation or targeting of other critical proteins involved in homeostasis. The early loss in cyclin marker in HT29 cell exposures (Figure 33), may be explained on the basis of cell-type susceptibility. Cyclin, also known as proliferating cell nuclear antigen (PCNA), accumulates in cells during G1 phase, is most abundant during S phase and it declines in G2 and M phases. It is known to be associated with DNA polymerase- δ involved in DNA replication (Kurki *et al.*, 1988). It could be that HT29 S-phase cells are the first affected by BT product, but there is no information regarding the differential susceptibility to BT of animal cells at different stages of the cell cycle (see Section 4.9).

The loss in genomic DNA integrity can be thought of as a late stage event of BT-induced damage, much like cytolysis. Time-course experiments indicate that during BT exposure, there was increased degradation of insect cell DNA (Figure 35). However, analysis

of the fragmentation patterns did not show the characteristic nucleosome-oligomer arrays seen in apoptosis of *Sf9* cells induced by superinfection with Hz-1 baculovirus (Lee et al., 1993) or HT29 human cells exposed to hypoxia (Yao *et al.*, 1995). Nuclease treatment of isolated nuclei from control *Sf9* cells has demonstrated detectable nucleosome oligomers (Figure 35B). When HT29 cells were exposed to BT, the remaining attached cells exhibited on occasion, nucleosomal-DNA oligomerization, but shed cells showed only extensive DNA degradation (Figure 36). The finding that DNA of attached cells is usually degraded could explain why these cells have no capacity to grow on replating. The excessive degradation of chromatin-DNA of shed cells may be caused by either endogenous nucleases or *Bt*-derived nucleases accessed after animal cells have lysed.

The survey of various human cell lines revealed that no cell type was impervious to BT-induced damage. However, one exceptional feature of Chang cells was the internalization of *Bt* cells (Figures 29 C, D and 30 F). This internalization of bacteria by Chang cells has not been seen until now. However, Chang cells are known to engulf dextran beads (Sit *et al.*, 1994). At present, it is not known if uptake of either *Btk* or *Bti* cells (as spores or vegetative cells) is due to engulfment by Chang cells or invasion by *Bt*. The engulfing capacity of Chang cells has been considered indicative of their macrophage-like liver function (Kupffer cells). Rappaport (1980) has shown by high speed video micrographs, that more than 99% of the bacteria that enter the liver via blood are removed by Kupffer cells. The rate at which a Kupffer cell internalizes a bacterium is ≤ 0.01 of a second (Rappaport, 1980). However, in contrast to the proposed *in vivo* process, *Btk* was not inactivated by Chang cells. Rather, they remained motile within the Chang cells for about two h after detection (ie. to the end of an

of cells to substrata is closely dependant on the association of the actin cytoskeleton with integral membrane proteins such as cell adhesion molecules (CAMs) and integrins (Baehni *et al.*, 1992). Since the actin immunoprobe detects both fibrous and globular actin (Lessard, 1988), the immunoblotting method might be useful to distinguish between cell detachment by depolymerization and rearrangement of the microfilament network. Detachment of cells from bioassay substrata has been seen for human gingival fibroblasts exposed to pathogenic *Treponema denticola* associated with periodontal disease (Baehni *et al.*, 1992). Here, the detachment of fibroblasts may be due to a trypsin or chymotrypsin-like protease that causes remodelling of the actin cytoskeleton into collapsed perinuclear fibers. In other studies, human skin fibroblasts exposed to *Candida albicans*, resulted in actin depolymerization followed by cell rounding and detachment from culture dishes (Marewicz *et al.*, 1995).

During insect and human cell exposure to either BT product or CF, both rounding and detachment were observed. Cytotoxicity was shown to be linked to the protein contents of *Btk* and *Bti* culture filtrates (CFs). The ³⁵S-methionine pulse-labelling experiments presented in Figures 73 to 75 show that in 10 min exposure to CF, both insect and human cells lose capacity to either take-up amino acids and/or synthesize proteins. The radiolabelled methionine incorporation of the first cells to round-up and to be shed was almost negligible. This indicates that these cells were not capable of protein translation. Alternatively, the cells may not be able to take-up nutrients like ³⁵S-methionine. In either case, cell detachment may ultimately result from a failure to replenish cellular proteins including actin.

4.4 Animal Cell Defence Responses

In the early stages of this thesis, experiments were conducted to determine if defence-like mechanisms such as those described in Table 1 and Figure 1 were expressed in either insect or human cell cultures. Testing of spent media produced by the various cell types, either in the presence or absence of BT, failed to negatively affect *Bt* growth in any demonstrable way. One possibility for the lack of growth inhibition has been because of *Bt* cell proteases such as “inhibitor A”, which is considered to be capable of degrading antimicrobial proteins like attacin and cecropin (Dalhammar and Steiner, 1984). Expression of the cecropin gene has been observed when a *Drosophila* blood cell line (mbn-2 cells) was stimulated with *E. coli* lipopolysaccharide, *Bt* subsp. *alesti* flagellin or *Micrococcus luteus* peptidoglycan (Samakovlis *et al.*, 1992). Although attempts to demonstrate antibacterial activity of animal cells in response to *Bt* were not successful, studies from the literature suggest that HT29 and other colon epithelial cell lines (Caco-2 and T84), actually increase transcript levels of several proinflammatory cytokines (interleukin-8, monocyte chemotactic protein-1, granulocyte-macrophage colony-stimulating factor and tumour necrosis factor α), on exposure to various bacteria (Jung *et al.*, 1995). Whether this expression affects *Bt* growth is currently unknown, but an unexpected result from thesis-related experiments, was that *Bt* production in DMEM was greatly enhanced, about 27-fold, by the presence of HT29 and other human cells. In fact, it was found that *Bt* spores germinated more efficiently in DMEM when human cells were present than when absent. This enhancement of *Bt* proliferation which did not occur in human cell preconditioned medium, further suggests that an actual “live cell” interaction may be necessary.

Besides cytokines and bactericidal proteins, epithelial cells are known to increase production of mucins, which immobilize bacteria and possibly prevent their spreading (Neutra *et al.*, 1987). Mucins are highly glycosylated, large proteins (>200 kDa) present on the apical surface of some polarized epithelial cells, especially insect midgut and human colonic goblet cells (Devine *et al.*, 1992). The accumulation of mucin may serve a protective role for animal cells as proposed for larval midgut cells exposed to *Bt* (Percy and Fast, 1983). We have observed that following exposures of ~4 h, MG1 cells may increase production of mucin from apical surfaces (Tayabali and Seligy, 1995). However, in other cell lines such as HT29, in which mucin is known to be produced by goblet cells within the monolayer, no increases in mucin production, or localized binding of *Bt* cells to HT29 cells were seen.

4.5 Effect of Temperature on BT Product Toxification of Insect Cells

The results presented in Figures 37 to 40 show that the major discrepancy between time of toxicity of insect and human cells by BT products could be accounted for by the difference in exposure temperatures used, and that this difference affects the rate of *Bt* proliferation and build-up of *Bt* cells (ie. dose-dependent lag). A comparison of data from published literature on *in vivo* toxification of insects from non-commercial *Bt* (Ignoffo, 1962; Raun *et al.*, 1966; van Frankenhuyzen, 1994), suggests that both *in vivo* and *in vitro* toxicity (data presented here) correlate directly with extent of *Bt* proliferation. At lower temperatures that may be encountered during spray operations (10 to 20°C), spore germination and cell proliferation would be retarded (Seligy *et al.*, 1997a). Under such conditions, the toxic contribution of δ -endotoxin may be more significant than that of the spore. The short half-life

observed to increase formazan production in response to low doses of whole BT product as well as CF (Figure 63C). From other studies, it is known that HT29 exposure to xenobiotics (Ciaccio *et al.*, 1994) or stress such as glucose deprivation (Lu *et al.*, 1992; Lu and Seligy, 1993), increases mitochondrial (dehydrogenase) gene expression.

When *E. coli* was used in similar experiments, the bioreduction decreased and levelled at ~49% of control (Figure 54 B and D). However, the *E. coli* dose used here, was four-fold greater than the highest dose of BT which resulted in a >90% loss in bioreduction activity. Scanning electron micrographs showed that numerous *E. coli* cells were present in the *Sf9* exposures, but none could be found in cultures of HT29 cells. Also, in comparison to control cells, both *Sf9* and HT29 treated cells appeared normal in morphology and could be passaged without cell loss. Unlike *B. subtilis* or *Bt* cells, *E. coli* produces MTT formazan at a low rate, so formazan accumulation did not increase during *Sf9* exposures.

In efforts to determine if the low toxicity from these bacteria was due to a growth-related effect, automated turbidity measurements were done (Figure 57). In the absence of *Sf9* cells (Grace's medium alone), growth of either bacterium was substantial compared to that when *Sf9* cells were present. However, in the case of human cell experiments, growth of either bacterium was very low in DMEM regardless of whether animal cells were present or not, but since some growth was detected, cultures derived from bacteria grown in the absence of animal cells were used in exposure experiments. Only cultures derived from *B. subtilis* grown for >12 h in Grace's medium were capable of causing cytotoxicity in four to six h exposures and the level of cytotoxicity corresponded to the accumulation of *B. subtilis* vegetative cells observed with turbidity measurements. *B. subtilis*-derived cultures were at

least 25-fold less cytotoxic compared to those of *Bt*. This difference was not due to a slow rate of bacterial cell accumulation because at 24-h there were 2.15 X more *B. subtilis* cells produced than *Bt* cells using identical inoculates. This means that the production of cytotoxic activity per *Bt* cell was twice as high as *B. subtilis* cells. Since *E. coli* cells were incapable of causing toxicity, the cytotoxic activity seen here may be bacterial species dependent.

4.7 Investigations into Possible Cytotoxic Constituents of Culture Filtrate

All evidence so far discussed, points to the exocellular byproducts produced soon after *Bt* germination and growth as the most toxic substance(s) for human and insect cells. To gain insight into what the toxic substance(s) might be, several different approaches were attempted. One set of experiments carried out by ³⁵S-radiolabelling of CF polypeptides demonstrated possible interactions between exocellular products of *Bt* and animal cells. The major putative binding component was a 30 to 32 kDa product (Figure 76) which corresponds to the molecular weight of *Bt* subsp. *gelechia* flagellin (Zhang *et al.*, 1993). Zhang and colleagues (1995) have recently shown that exposures of *Sf9* to purified *Bt* flagella, resulted in high levels of binding accompanied by cell death. If antiserum directed towards flagella was included in the exposures, killing was abolished. However, the specificity of the antiserum is questionable because it was actually made from a crude preparation of flagellin and most probably cross-reacted with other exocellular vegetative cell proteins. It is reasonable to suggest that any cytotoxins present would have been inhibited during the co-incubation with the polyclonal antibodies present. An earlier study demonstrated that mutants of the *Bt* subsp. *gelechia* which are incapable of motility, were

2500 to 7500 -fold less toxic to *T. ni* larvae, based on LD₅₀ (Zhang *et al.*, 1993), but the mutants were known to be pleiotropic, exhibiting several differences compared to the wild-type strain. Flagella have long been thought of as structures responsible for bacterial motility, but binding of flagella to animal cell membranes may be critical for increasing the local concentration of cytotoxic factors produced by vegetative cells (Zhang *et al.*, 1995). During the ³⁵S-labelled polypeptide experiment, several other CF products were found to also bind to Sf9 and HT29 cells (ie. 15, 22, 40, 45 and 80 kDa), any of which may correspond to the CF toxic constituent. In order to identify the toxic constituent of BT products, several candidates could either be directly purified from BT products (eg. the δ -endotoxin-containing PIB) or obtained from growth cultures (eg. VIP). Commercially available forms of PI-PLC and BCET were also examined for their ability to cause cytotoxicity similar to that of CF.

4.7.1 Parasporal Inclusion Body-associated Toxicity

The toxicity characteristics induced by exposure to whole BT products and CF described in sections 3.4, 3.5 and 3.10, resembled published claims for toxicity caused by a variety of agents such as bacterial cells, flagellin, activated δ -endotoxin and VIP3A (Estruch *et al.*, 1996; Himeno *et al.*, 1985; Murphy *et al.*, 1976; Nishiitsutsuji-Uwo and Endo, 1979; Thomas and Ellar, 1983; Yoshida *et al.*, 1993; Yu *et al.*, 1995; Zhang *et al.*, 1993). A few of the publications listed, describe assays to assess effects from δ -endotoxins prepared by different methods. Some of these studies have used Sf9 as the target cell, but none have actually purified or enriched for PIBs directly from commercial BT products. When activated CryIC was used, the first change detected in Sf9 cells was an immediate, but non-toxic rise in intracellular Ca⁺⁺ (Monette *et al.*, 1997). This change in Ca⁺⁺ flux was speculated to

represent toxin docking and intercalation into the cell membrane. Following the initial rise in intracellular Ca^{++} , there was the time-dependent appearance of anion (chloride) selective pores and increased membrane permeability to K^+ , H^+ and Na^+ within 5 to 10 min (Schwartz *et al.*, 1991). Using a variety of activators and inhibitors of Ca^{++} flux that affect movement either across the plasma membrane or intracellular membranes (eg. mitochondria or endoplasmic reticulum), Monette and colleagues (1997) have shown that CryIC toxicity is dependent on both extracellular and intracellular Ca^{++} levels. Results from measuring uptake of ethidium homodimer as an indicator of loss in membrane integrity revealed that the LD_{50} for CryIC was 14 $\mu\text{g/mL}$ with a 3-h exposure. Another study using the same δ -endotoxin preparation, gave an LD_{50} of 125 $\mu\text{g/mL}$ in 2.5 h using trypan blue viability assays (Smith *et al.*, 1996). The difference in LD_{50} values may be due to the sensitivities of the two cytotoxicity assays.

Using the ethidium homodimer assay, toxicity of δ -endotoxins CryIAa, CryIAb, CryIAc, CryIIA and CryIIIA, have also been evaluated with *Sf9* cells but they were shown to be about 8-fold less toxic compared to CryIC δ -endotoxin (Monette *et al.*, 1997). The presence of pro- δ -endotoxin genes (*cryIAa*, *cryIAb*, *cryIAc*) within BT products (ie. spores) has been confirmed (Seligy *et al.*, 1997a; Seligy *et al.*, 1997b; Tayabali and Seligy, 1995). Furthermore, SDS-PAGE analysis of BT products (Figures 5, 44 and 45) suggested that there is a large amount of activated (62 to 67 kDa) δ -endotoxin protein present (~20 % of total). Yet, when exposures were conducted even over a 24-h interval, only moderate toxicity was observed. Preparations of *Bti* also contained large amounts of CytA, but given the close comparison between toxicity profiles of *Btk* and *Bti* -BT products (with or without

gentamicin), CytA really did not have much of an additional effect (if any) at the doses examined. Based on calculations for BT product high dose (10^{-3} I.U./cell), there should have been about 8 $\mu\text{g/mL}$ of *Bti* PIB and 17 $\mu\text{g/mL}$ of *Btk* PIB present. In contrast, BT products without antibiotic or BT products digested with a broad-spectrum protease to remove all PIB-associated proteins from spores, produced an extensive cytotoxic response at either 27°C or 37°C. It is possible that both the activated δ -endotoxin and pro- δ -endotoxin in BT products are inaccessible as they are very non-antigenic using murine response models (Bernstein *et al.*, 1997; Seligy *et al.*, 1997b).

Several publications have described methods for preparing “ δ -endotoxin”, “crystal toxin” or “crystal proteins” (Percy and Fast, 1983; Singh *et al.*, 1986). These preparation/purification methods are quite mechanical and crude and do not include precautions to eliminate spores, which contribute to toxicity in the insect (van Frankenhuyzen, 1994). For advanced methods involving HPLC, the matter of contamination may be less because of filtration. In the PIB enrichment scheme used in this thesis, precautions were taken to prevent spores from acting on animal cells by use of antibiotics. Following solubilization of the PIB preparations and cleavage of pro- δ -endotoxin by trypsin, the samples were centrifuged and the supernatant fraction was slowly passed through a 0.45 μm filter. This method is routinely used to sterilize media and to remove *Bt* spores which measure $\sim 0.75 \times 1.2 \mu\text{m}$. Spread-plating of various δ -endotoxin preparations at 1X exhibited no bacterial colonies after 48 h of incubation. As a further precaution, all PIB exposures were done in the presence of gentamicin. Careful monitoring of assays by light microscopy and SEM during exposure regimes (Figures 49 and 50) did not reveal presence of *Bt* spores or vegetative *Bt*.

Results from doses of 100 $\mu\text{g/mL}$ of the spore-free *Btk* PIB preparations gave only 10 to 28 % decreases in bioreduction of *Sf9* and HT29, respectively. In contrast, the enrichment of *Bti* PIB gave an LD_{50} of 40 to 57 $\mu\text{g/mL}$ for both cell lines. The higher activity of *Bti* PIB may be related to the CytA toxin, which by SDS-PAGE was present in the PIB preparation at $\sim 10 \mu\text{g/mL}$. Thomas and Ellar (1983) also observed morphology changes such as cell rounding, swelling, blebbing and lysis of *Sf9* exposed to 20 $\mu\text{g/mL}$ alkali-solubilized *Bti* PIB for 40 min and mammalian cells (mouse fibroblasts and epithelial carcinoma cells and primary pig lymphocytes) exposed to 25 $\mu\text{g/mL}$ for 30 min. It is possible that the toxic activity of solubilized *Bti* PIB may be due to carry-over of VIP3A-like, CF activity produced during vegetative cell proliferation in the preparation of *Bt* toxins.

The toxic effects of the *Bti* PIB preparations were not as severe as CF from vegetative cells harvested at 6 to 8 h of BT product cultures. CF tested in bioassays gave an LD_{50} of one and five $\mu\text{g/mL}$ in two h assays with HT29 and *Sf9*, respectively. Since PIBs may be produced during sporulation phase beginning at about 18 h or later, the activity of the toxic ingredient(s) in CF should actually be higher when harvested at late incubation times. However, this was not the case and PIB proteins did not accumulate as a dominant component in stained SDS-PAGE gels of CF (Figure 68). Collectively, these observations show that BT product toxicity is not primarily due to the δ -endotoxin, but rather to another growth-related component.

4.7.2 Toxicity of Phosphatidylinositol Phospholipase C (PI-PLC)

Several bacteria rely on phospholipases as part of their repertoire of virulence factors for pathogenicity. Two bacteria that are closely related to *Bt* (*B. cereus* and *B. anthracis*) are

exposed to this PI-PLC was lower than that of untreated cells, but the plateau of ~65 % relative to untreated cells suggests that PI-PLC caused limited injury. This is a reasonable claim since there were no changes in the levels of attached cell number and markers such as actin and hsp70. Also, cells could be passaged after PI-PLC treatment. It may be that higher doses of PI-PLC are needed, but Ikezawa and colleagues (1989) obtained an LD₅₀ for TN-368 cells with 0.2 units/cell. Zhang and colleagues (1985) also failed to see any gross cytotoxic response in mbn-2 *Drosophila melanogaster* cells exposed to one unit / mL using trypan blue as an indicator. Collectively, the assays conducted here and reported by others all indicate that at the macro level, PI-PLC is not a major toxification factor of either insect or human cells in CF. However, it is possible that PI-PLC may act synergistically with several substances to cause the dramatic effects of the CF as seen in this work.

4.7.3 *Bacillus cereus*-like Enterotoxin (BCET)

The 5.6 megabase chromosomes of *B. cereus* and *B. thuringiensis* are very closely related and include genes with virulence functions (Ash *et al.*, 1991; Carlson *et al.*, 1996; Nakamura, 1994; see review in Seligy *et al.*, 1997b). It is proposed that *B. cereus* produces two key enterotoxins: an emetic exotoxin and a diarrhoeal enterotoxin. Neither of these factors has been characterized in much detail. The presence of diarrhoeal enterotoxin (BCET) correlates with health effects exhibited between 8 to 16 h with a total oral dose of 10⁴ to 10⁷ cells or spores per human adult (Kramer and Gilbert, 1989). Given the close genetic relationship of *Bc* and *Bt*, and that commercial BCET tests are positive for BT products (Beggs *et al.*, 1996), it was important to determine whether BCET activity could mimic the CF toxicity produced from BT products. This was accomplished by testing the BCET

standards from commercial enterotoxin test kits in insect/human cell bioassays. Of the two kits, only the Tecra kit standard generated a loss in bioreduction activity in both *Sf9* or HT29 tester cells (Figure 59), but in either case there was no change in cell attachment from well surfaces. With SEM, some damage to *Sf9* membranes was seen in the form of lesions (Figure 60D). This type of damage was clearly distinct from the damage caused by CF at high doses. The HT29 cells also showed no major membrane lesions, but about 60% of the *B. cereus* enterotoxin-treated cells exhibited multiple pseudopod-like extensions (Figure 61D). At low doses of CF, neither bioreduction nor cell surface morphology changed. Efforts to characterize the toxic component of the BCET commercial standards were hampered by protein yield and the excessive amounts of the 68 kDa BSA carrier present. The BSA itself does not promote any signs of cytotoxicity in the insect/human bioassays. From the literature, the molecular weight of the toxin should be 40 to 45 kDa (Asano *et al.*, 1997; Granum *et al.*, 1993). Since the standards from Oxoid and Tecra BCET commercial test kits exhibited no reciprocal cross-reactivity, it is doubtful that they are the same.

4.7.4 Vegetative Insecticidal Protein (VIP)

Several groups have now reported finding other insecticidal agents produced by *Bt* (Asano *et al.*, 1994; Estruch *et al.*, 1996; Walther *et al.*, 1986; Yu *et al.*, 1995; Zhang *et al.*, 1993). These investigations have all been recent and focussed on vegetative cell exoproteins. In particular, a patent has been recently filed describing the analysis of VIP3A, a novel insecticidal protein produced by several *Bt*-related organisms (Warren *et al.*, 1996). When purified and fed to black cutworm larvae, VIP3A was found to be ~260-fold more potent than Cry1A(a) or Cry1A(c) δ -endotoxins (Yu *et al.*, 1995). All larvae died within 72-h exposure

to 62 ng of VIP3A per mL of diet. Unfortunately, the actual absorbed dose is not known and LD₅₀ values for cells of insects and other animals have not been determined.

The experiments leading to the discovery of cytotoxic CF reported in this thesis, were independent of the work of others and directly evolved from studies on BT product exposure investigations. What was discovered is that crude unfractionated exoproteins of CF produced by vegetative cells of either *Btk* or *Bti*, gave an LD₅₀ for either insect or human cells of five and one µg/mL, respectively in two h exposures (Figure 70B). The changes to the morphology of the cells in the larval midgut following VIP3A administration (Yu *et al.*, 1997) are remarkably similar to those illustrated in Figures 64, 65 and 66 for MG1, Sf9 and HT29 cells. Yu and colleagues show that within 24 h, the midgut columnar cells of the black cutworm larva were swollen, but the goblet cells appeared normal. However at 48 h, gut lumens were filled with debris and shed cells and by 72 h, the midgut epithelium was destroyed and no longer identifiable. The gene encoding VIP3A has been sequenced and is not related in any way to δ-endotoxin genes or to other bacterial toxins (Estruch *et al.*, 1996). It is possible that a part of the cytolytic activity of *Bt* toxin purifications, including PIB preparations, is due to carry-over of CF cytolytic activity. The survey by these authors indicate VIP3A was found only in select strains of *Bt*. However using polymerase chain reaction, the VIP3A gene(s) have been detected in spores of all BT products and *Bt* vegetative cells derived from spores (Seligy *et al.*, 1997b). The VIP3A protein has a relative molecular weight of 80 kDa and can be reduced with chymotrypsin or larval midgut proteases to fragments of 22, 33, 45 and 62 kDa. All of the cleavage products were toxic to several different types of larvae including those of *Spodoptera* species (Estruch *et al.*, 1996; Warren

1995). The ADP-ribosylation of a target protein renders it inactive through the addition of ADP-ribose to amino acids arginine, asparagine or cysteine. In *in vitro* assays with platelet membrane preparations, an exo-ADP ribosyltransferase from *B. cereus* culture filtrates has been reported to ribosylate “rho”, a protein responsible for controlling actin polymerization/depolymerization (Just *et al.*, 1992). This same group, and also Krueger and Barbieri (1995) have shown that the unrelated ADP-ribosylating toxins of *Clostridium botulinum* transfers the ADP-ribose moiety of NAD to globular actin (42 kDa), resulting in the net depolymerization of microfilaments. Toxins of *Vibrio cholera* and enteropathogenic *E. coli* ribosylate G-proteins causing intracellular accumulation of cyclic AMP and subsequent electrolyte losses (Rappuoli and Pizza, 1991). Based on studies comparing the structure and function of ADP-ribosyltransferases from avirulent mutant organisms, active toxins are often oligomeric with different subunits functioning in cell membrane binding, translocation and enzymatic activity. At this stage, it is not certain whether the 45 kDa ³²P-labelled polypeptide of CF can ADP-ribosylate animal cell proteins. However, the NAD covalent interaction of *Btk* or *Bti* CF is labile since several experiments showed that labelling levels decreased after 80 min of incubation (Figure 85B). Exclusion chromatography suggests that the ³²P-product chromatographs with one broad cytotoxic activity peak. It is not clear if it is an auto-ribosyltransferase or whether the transferase activity is mediated by other CF components. The bioassays combining different column fractions showed no synergism.

4.8 Relevance to BT Exposure *in vivo*

Although the central goal of this thesis was not directly concerned with either BT product efficacy or improving our understanding of target specificity, and also possible health and safety issues, important new baseline information on these BT products has been produced. The following two sections are intended to put some of the results in perspective with environmental field application of BT product.

4.8.1 Improvement of Product Efficacy

In Canada, commercial *Btk*-BT products are most often applied by aircraft as aerosols in remote forest areas, but in the USA they are also sprayed from the ground over plants used in agri-food production (Seligy *et al.*, 1997b). In either case, droplets of *Btk*-BT product are deposited on foliage for protection against target insect larvae. For *Bti*-BT products, they are injected or sprayed into streams or shallow bodies of water, where mosquito and blackfly larvae develop. For either application approach, dose acquisition by target organisms is a crucial step, and rates of consumption of BT product deposits are often highest only at crucial stages of development (Nealis *et al.*, 1992). For example, during the first and second instar, a cabbage looper consumes about 2.2 cm² of leaf surface (Ignoffo *et al.*, 1974). Following a typical spray application of 30 to 40 billion I.U./hectare, 30 to 40 I.U./cm² or 72,000 to 103,200 cfu/cm² of leaf or collector surface has been detected (Dunbar *et al.*, 1973; Seligy *et al.*, 1997b). This means that each larva, depending on species, might consume 66 to 94.6 I.U. or at least 1.6×10^5 spores, based on our estimate of ~2400 spores/I.U. (Seligy *et al.*, 1997a). This calculation assumes that cessation of feeding does not occur, even though it often does (Payne and van Frankenhuyzen, 1995).

Once taken within the larva, the general assumption is that only the *Bt* PIBs act as a toxin and that this is done by solubilizing the PIB in the specific alkaline environment of the midgut, and that the pro- δ -endotoxin released by solubilization would be cleaved by midgut proteases. The “activated” δ -endotoxin molecules are then believed to cause midgut cell disruptions as outlined in the literature (section 1.4). Prior to this epithelial disruption, it is assumed that the alkalinity of the gut would prevent *Bt* spores from germinating, but this may not be the case as high pH can also activate *Bt* spores (Seligy *et al.*, 1997a; Wilson and Benoit, 1993). From the *in vitro* bioassays conducted here, spore activation leads to germination and cell amplification for continuing the life (growth) cycle. Recent work by Yu *et al* (1997) suggests that following δ -endotoxin-induced midgut disruption, spores may germinate and cause wide-spread septicaemia and larval death at about 16 to 24 h.

Until very recently, there has been little mention of virulence-enhancing factors produced by the *Bt* vegetative cells during infection such as described in this thesis and those like *B. cereus*-like enterotoxin, PI-PLC, proteases and VIP3A (Beggs *et al.*, 1996; Damgaard, 1995; Estruch *et al.*, 1996; Tayabali *et al.*, 1996; Tayabali and Seligy, 1997). It is likely that the ability of biological control products of this type to cause mortality *in vivo* would depend on the synergistic actions of PIB proteins and spore growth products. In studies from the literature, whenever live spores were administered to larvae (with/without PIB), the cadaver had large numbers of vegetative bacilli in their midgut and haemocoel (Aly, 1985; Ignoffo, 1977; Sutter and Raun, 1967; van Frankenhuyzen, 1994). In fact, a single spore ingested by a larva is sufficient to cause death of even relatively resistant insect species such as *Spodoptera* (Zhang *et al.*, 1993). Also, when *Bt* subsp. *gelechia* strains lacking δ -endotoxin

were fed to *T. ni* larvae, infection and death resulted (Zhang *et al.*, 1993).

Using the data from the *in vitro* bioassays described in this thesis, it is possible to show that such bioassays are consistent and predictive of whole animal mortality (LD_{50}) for a wide range of doses and temperatures (23 to 37°C). These thesis findings and conclusions have now been recently published (Tayabali and Seligy, 1997). Although the lowest temperature used for these assays was only 23°C, our laboratory has shown that *Bt* growth can occur at temperatures as low as 12.5°C, which could be encountered under outdoor field conditions in May to June (Seligy *et al.*, 1997a). Since insect cell mortality has been shown to be directly related to the rate of vegetative cell accumulation, the time for LD_{50} of larvae would be consistent with changes at both low and high temperatures.

4.8.2 Impact on Health and Safety

Similar to insect larvae, humans may internalize BT by routes such as ingestion and inhalation, and through dermal abrasions. The results here suggest that the most dangerous situation would arise when spores are able to germinate and accumulate as vegetative cells, which is essentially an infection. It has been recently shown that *Btk* or *Bti* vegetative cells grow optimally in the range of human body temperature ($37 \pm 2^\circ\text{C}$) and pH, and even at elevated temperatures that may be encountered during a fever (up to 40°C) (Seligy *et al.*, 1997a). The main advantage humans (and mammals) have in managing onslaughts of infection is the elaborate cell-based immune system, which acts in concert with localized, tissue defence mechanisms to allow tolerance to even relatively high transient doses of microbes. Most BT exposures encountered by occupational workers occur over a short period of time (less than one h) within one season and in vast, open spaces (Seligy *et al.*,

cereus and that they are capable of producing potent cytolytic toxins if infection is allowed to occur.

4.9 Summary and Future Studies

Over the last 10 years, several BT products have been commercially developed for current large-scale production and use. All these products are manufactured outside Canada and detailed published information on the toxicity and compositions of the active ingredient of these BT products has been largely unavailable until very recently. This thesis provides details on: (1) the use of multiple bioindicator assays to compare, in a standard way, animal target cell responses to commercial microbe-based biotechnology products, (2) to obtain toxicological data relating BT dose-time effects on insect and human cell systems, and (3) to identify the possible hierarchy of cytotoxic components, the “active ingredients” of BT products, bearing in mind their crudeness and complexity.

The *in vitro* assay systems developed and optimized here involved the use of multiple indicators such as cell morphology, bioreduction activity, immunodetection of several proteins, *in vivo* protein synthesis and DNA integrity, and several commercially available insect and human cell systems. The emphasis on using commercially available assay components was deliberate to allow others in the toxicology field to readily set-up similar test systems.

Since no information about BT products was available from published literature at the start of this thesis, our laboratory provided critical information on their major constituents, which are mainly viable spores, proteinaceous granules and to a lesser extent, intact PIBs.

gentamicin also showed extensive cytoplasmic disruptions. Those cells that appeared to remain intact were non-viable on subsequent passage. A novel response of the human (Chang) liver cell line was the internalization of *Bt* cells. The reasons for this internalization of motile, vegetative bacteria remain unclear. Chang cells are known to internalize inert matter and may reflect a Kupffer macrophage cell-like origin. The shed insect and human cells exhibited heavily vacuolarized cytoplasm and pyknotic nuclei, indicative of irreversible injury resembling programmed cell death. The loss in protein markers associated with lysed cells was >99%. The differential decrease in cyclin seen in human colonic cells (HT29) suggests that a cell cycle-related susceptibility to cytotoxic action may occur. In all cases, irreversible target cell DNA degradation resulted from these exposures. This degradation appeared to occur late, and only HT29 cells occasionally showed the nucleosomal DNA fragmentation characteristic of mammalian apoptosis. However, it was shown that insect cells had the potential to generate nucleosome-related fragmentation patterns using an exogenous nuclease assay, but this pattern of degradation was not seen with exposure to either whole BT product or vegetative cell by-products.

The pattern of cytotoxicity of insect and human cells exposed to BT products differed only in their time of response. For doses spanning four orders of magnitude, insect cells had half-lives between six and 15 h, whereas human cells had half-lives between 4.5 to 6 h. This discrepancy was shown to be due to temperature (27°C versus 37°C), which controls the rate of *Bt* vegetative cell production. Several tests with washed vegetative *Btk* cells revealed that the toxic response was caused by extracellular products expressed relatively early after spore germination (ie. six h with FBS present or eight h without FBS present). Exposure to cell-

cell cultures from various target tissues or whole animals such as those described in recent work by Peyronnet and colleagues (1997). Also, it is not known whether differentiated cells have an altered susceptibility to BT. To this end, cell lines such as HT29 may be useful to model BT effects on colonic epithelial differentiation. Also, the reason for K562 cell resistance to BT could be investigated since almost no marker losses were seen during exposure. Little is known about the exact mechanism of marker loss, other than that resulting from cell lysis. Microfilament (fibrous actin) depolymerization and rearrangements may be mapped in BT exposures as has been described in *in vitro* exposures of epithelial cell monolayers to enteropathogenic bacteria such as *Escherichia coli*, *Treponema denticola* and *Helicobacter pylori* (Baehni *et al.*, 1992; Jepsin *et al.*, 1993; Smoot *et al.*, 1993). Heat shock protein studies have shown that following exposure to various stresses, hsp 72 and 73 relocate into different subcellular compartments (Komatsuda *et al.*, 1993). Immunolocalization studies with fluorescence microscopy may provide the critical intracellular location of biomarkers and cytotoxic constituent(s) during response to BT and CF exposure. As a marker for cell proliferation, the rapid decrease in cyclin level suggests an S-phase sensitivity to toxic action. This could be tested by monitoring status of ³H-thymidine labelled cells using *in situ* autoradiographic detection during exposure to BT and CF. Currently, there is no published literature on gene expression (transcript) levels of these and other indicators. This aspect can be investigated by using either specific RNA probes (actin, cyclin, hsp70) or *in vitro* translation of messenger RNA followed by fractionation of polypeptides by PAGE and autoradiography or immunoprecipitation. In preliminary thesis work, such experiments were attempted but no results were obtained, possibly because RNA

loss was rapid. The various types of approaches described here may be able to focus on the action of the cytolytic component(s) and possible ADP-ribosylation of various key proteins. A unique response of one cell system, Chang liver cells, was that they showed internalization of vegetative bacteria. It is not known whether this is a general response of all phagocytic cells. This could be tested using macrophage from lung lavages or white blood cells harvested from blood.

The bioassays described here provide comparable results to *in vivo* insect larval assays (Ignoffo, 1962; Raun *et al.*, 1962; van Frankenhuyzen, 1994) in the temperature range tested here between 23°C and 37°C. However, *Bt* germination and growth have been observed in the range of 12.5 to 42°C (Seligy *et al.*, 1997a) which suggests that further investigations could be done to extend the outer temperature range to reflect environments encountered by these bacteria during spray season and fever temperatures. The choice of bioassays described here are readily applicable for testing a variety of other biopesticide products including those formulated with PIB crystalloids encapsulated in killed heterologous cells like *Pseudomonas* (ie. "MPV" product) and also nucleopolyhedrosis virus (ie. "Gyptex" product).

Finally, this study has uncovered new cytolytic toxins which are associated with organisms of BT products. The results presented here suggest that the *Btk* and *Bti* cytotoxic agent(s) resembles the VIP3A protein, recently reported by one group (Estruch *et al.*, 1996; Yu *et al.*, 1995). However, at present we know less about the mechanism of action of VIP3A than the CF cytotoxic substance(s) identified here. In either case, it would be useful to demonstrate if there is molecular equivalence between VIP3A and the CF cytotoxic substance(s). This could be done by molecular cloning or purification using large-scale

methods and amino acid sequence analysis. Since the CF toxic substance has been shown here to be harmful to both insect and human cells, the suggested use of VIP3A as a novel insecticidal product (patent pending: Warren *et al.*, 1996) should be challenged as it may be a serious hazard to humans and should be assessed early, before much further effort is expended.

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