# The Efficacy of Vaporous Hydrogen Peroxide and Gaseous Chlorine Dioxide in the Detoxification of Ricin and *Bacillus anthracis* Lethal Factor and Protective Antigen

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## Abstract:

Biological toxins such as ricin and the *Bacillus anthracis* toxins have become an increasing bioterrorism threat due to the relative availability, extreme potency and ease of production of these agents. Extensive research has been conducted investigating methods for decontaminating spaces affected by biological agents such as bacteria and viruses, but little research has been done to determine the efficacy of these methods on toxins. The efficacy of gaseous chlorine dioxide (GCD) and vaporous hydrogen peroxide (VHP) at inactivating ricin and *B. anthracis* lethal factor and protective antigen was tested. The presence and activity of the toxins after exposure to the decontamination methods was detected using a cytotoxicity assay and protein gel electrophoresis. Both VHP and GCD were found to be effective at detoxifying the anthrax toxins within a short exposure, with close to complete inactivation observed during longer exposures. Ricin proved to be more resistant to inactivation, with longer exposures needed to achieve similar levels of detoxification to the anthrax toxins. Overall, GCD and VHP have great potential for use in inactivating biological toxins.

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## List of Abbreviations:

AC- adenylyl cyclase

ADP- adenosine di-phosphate

ANTXR1/2- Anthrax toxin receptor 1/2

ASTM- American Society for Testing and Materials

ATP- adenosine tri-phosphate

AtxA- Anthrax toxin activator

BSA- bovine serum albumin

BSC- Biological Safety Cabinet

BWC- Biological Weapons Convention

cAMP- cyclic adenosine monophosphate

CaM- calmodulin

CARD- caspase activation and recruitment domain

CDC- Centre for Disease Control

CL3- Containment level 3

CMV- Cytomegalovirus

DCs- dendritic cells

DMEM- Dulbecco's Modified Eagle's Medium

DNA- Deoxyribonucleic acid

dNTPs- deoxyribonucleotide triphosphates

DRDC- Defense Research and Development Canada

ECV- endosomal carrier vesicles

EDTA- ethylenediminetetraacetic acid

EF- Edema Factor

ELISA- enzyme-linked immunosorbent assay

EM- Electron Microscopy

EPAC- guanine nucleotide exchange factor for Ras GTPase homologs

ERAD- ER-associated protein degradation

ERK- extracellular-signal-regulated kinase

ER- endoplasmic reticulum

FBS- Fetal Bovine Serum

GCD- Gaseous Chlorine Dioxide

GFP- green fluorescent protein

GTPase- guanine tri-phosphatase

IE- immediate early

IL-1β- interleukin 1β

IL-6- interleukin 6

IL-12- interleukin 12

JNK- c-Jun-N-terminal kinase

KGB- Soviet Union Committee for State Security

LB- Luria-Bertani

$LD_{50}$- lethal dose 50

LF- Lethal Factor

LRP6- lipoprotein-receptor-related protein 6

MAPK- mitogen-activated protein kinase

MAPKK- mitogen-activated protein kinase-kinase

MEM- Minimal Essential Medium

MIDAS- metal ion-dependent adhesion site

MKKK- mitogen-activated protein kinase kinase kinase

mRNA- messenger RNA

MTT- 2,5-diphenyl-tetrazolium bromide

MVB- multivesicular bodies

NEB- New England BioLabs

NIH- National Institute of Health

NLR- nod-like receptor

NML- National Microbiology Laboratory

NSF- National Sanitation Foundation

ORF- Open Reading Frame

PA- Protective Antigen

PABD- PA binding domain

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PDI- protein disulfide isomerase

Phe clamp- Phenylalanine clamp

PNMs- polymorphonuclear cells

ppm- parts per million

QCT- Quantitative Carrier Test

RCA- *Ricinus communis* agglutinin

RH- Relative Humidity

RIP- ribosome inactivating protein

RNA- Ribonucleic Acid

rRNA- ribosomal RNA

RTA- ricin toxin A chain

RTB- ricin toxin B chain

SARS- Severe Acute Respiratory Syndrome

SASPs- Small Acid Soluble Proteins

SDS- Sodium Dodecyl Sulfate

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBE- Tris-Borate EDTA

Th1- T helper cells

TNF-α- tumor necrosis factor α

TLRs- toll-like receptors

UV- Ultra Violet

VHP- Vapourous Hydrogen Peroxide

VWA- von Willebrand factor type A

# CHAPTER 1.0: INTRODUCTION

## 1.1 *Bacillus anthracis* and the Anthrax Toxins

### 1.1.1 Introduction to *Bacillus anthracis*

*Bacillus anthracis* is a gram positive spore forming bacterium and the etiological agent of anthrax disease. As a member of the *Bacillus cereus* group (*B. cereus, B. thuringiensis, B. mycoides, B.anthracis and B.weihenstephensis), B. anthracis* exhibits very similar growth characteristics and genetic material to other members of this group. As with other *Bacillus* species, *B. anthracis* is readily found in the environment and is able to survive in soil, water and vegetation. Vegetative cells are square end rods and range from 3-8 μm in length making them one of the largest of the pathogenic bacteria (Bhatnagar and Batra 2001; Koehler 2009).

Anthrax disease has been closely linked with human history for centuries. It is considered to be one of the Egyptian plagues during the time of Moses and was described in early Hindu and Greek literature (Bhatnagar and Batra 2001). The disease was historically known as woolsorter's disease and ragpicker's disease as it was associated with the inhalation of dust containing *B. anthracis* spores during the processing of animal wool and hides. *B. anthracis* was important historically as the first bacterium known to cause a disease. As the model for Robert Koch's postulates of disease, *B. anthracis* became mankind's first proven germ (Bhatnagar and Batra 2001).

### 1.1.2 *Bacillus anthracis* endospore

The *B. anthracis* endospore is the predominant form outside of the host and the infectious particle of anthrax disease. It is considered to be an ideal infectious agent as this dormant form

is highly resistant to starvation and can survive in hostile environments until the opportunity for infection arises (Driks 2009). *B. anthracis* spores have been found to survive in the soil of many diverse climates for decades. Once inside the host, the spore is able to survive host defenses allowing the bacteria to colonize its host.

Sporulation is a complex process involving a series of coordinated steps that can take up to eight hours to complete (Driks 2009). The process is initiated by low nutrients although the exact chemical signals that trigger sporulation remain unknown. Extensive studies in *B. subtilis* have revealed an elaborate signal transduction network including a variety of signals such as nutrient level, cell cycle state and secreted factors, responsible for controlling sporulation (Driks 2009). In sporulating cells the forespore is approximately 1-1.5um in diameter and can form either in the centre or subterminal end of the mother cell (Koehler 2009).

The spore consists of a series of concentric layers (Figure 1.1.1), with the inner most (core) containing the tightly compacted chromosome and small acid soluble proteins (SASPs). SASPs interact with the DNA and along with calcium dipicolinic acid prevent DNA damage from heat, UV radiation and other stresses (Driks 2009). The next layer, the cortex, is composed of peptidoglycan and assists in keeping the core dry. Surrounding the cortex is the coat, a multilayered shell containing ridges and valleys that provide flexibility necessary to allow for the increase in volume during germination. The coat also has a protective function in that is prevents the entry of large degradative molecules as well as the toxic activities of smaller reactive molecules that could damage the inner layers (Driks 2009). The outermost layer (exosporium) of the *B. anthracis* spore is separated from the coat by a gap called the interspace and is only present in some *Bacillus* species including the *B. cereus* group (Driks 2009). The

exosporium is not present in the more extensively studied *B. subtilis* and is believed to play a role in the interactions between the spore and its environment (Driks 2009; Koehler 2009). Once inside the host, or when more favourable conditions are encountered, the spore will begin to germinate. Germination is triggered by amino acids, ribonucleotides and peptidoglycans, collectively termed germinants. Germinant binding to receptors in the spore inner membrane activates a family of *ger-A* like sensor operons, which leads to a cascade of events beginning with an influx of water causing the spore coat to swell (Liu, Bergman et al. 2004; Driks 2009). After this the cortex and the coat are dismantled followed by the resumption of metabolic activity and cell growth, known as outgrowth (Driks 2009).

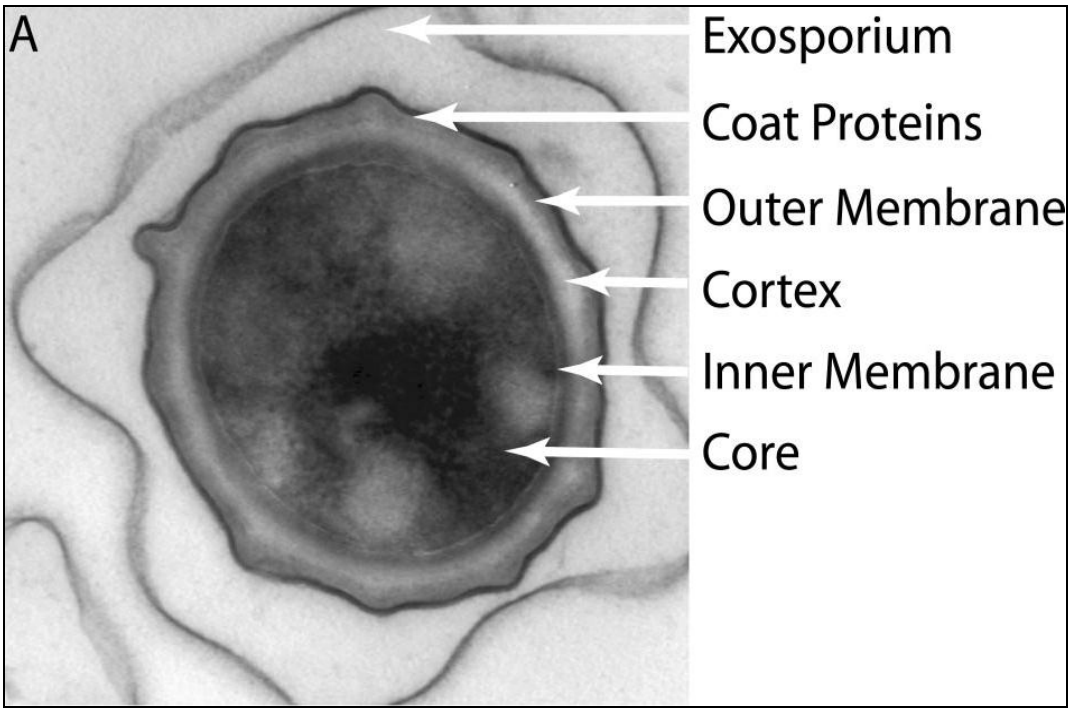


Figure 1.1.1: Transmission electron micrograph of *Bacillus anthracis* spore.

Reprinted with permission from American Society of Microbiology: Journal of Bacteriology (Liu, Bergman et al. 2004).

### 1.1.3 Anthrax Disease

Anthrax disease has been reported all over the world and is still endemic in many developing countries. In nature the disease is primarily associated with herbivores, especially cattle and sheep, which ingest the spores while eating grass. Human cases of anthrax disease are usually transmitted from these lower animals through contact with meat and animal byproducts rather than from infected humans. It is believed that human to human transmission may be possible based on evidence from an outbreak of epidemic cutaneous anthrax in Gambia (Bhatnagar and Batra 2001). In Europe and North America, cases of anthrax disease are most commonly connected with the processing of animal hair, wool, hides and bones, while in Asia and Africa the disease is often contracted from contact with diseased domestic animals (Bhatnagar and Batra 2001).

The symptoms and disease progression of anthrax varies based on the mode of infection. Three different forms of human anthrax disease can occur: cutaneous, gastrointestinal and inhalational. Diagnosis of the disease can be difficult, as the early symptoms are non-specific, and the diagnosis is generally achieved by detection of *B. anthracis* in clinical samples.

#### 1.1.3.1 Cutaneous anthrax

Cutaneous anthrax is the most common form in humans and occurs when spores from the environment or infected animals contaminate skin wounds. Within a week of inoculation the contaminated skin lesion develops into a papule, after which small translucent vesicles containing bacteria form around the papule, accompanied by massive edema (Bhatnagar and Batra 2001; Baldari, Tonello et al. 2006). As tissue necrosis worsens, the skin vesicles rupture resulting in the release of bacteria and formation of black eschars, the scabs characteristic of

cutaneous anthrax. The black eschars are the basis for the name anthrax which is Greek for coal. Cutaneous anthrax can be successfully treated with antibiotics, but left untreated severe forms can result in toxemia, sepsis and death (Bhatnagar and Batra 2001).

#### 1.1.3.2 Gastrointestinal anthrax

Gastrointestinal anthrax is less common and develops following ingestion of contaminated meat products. A few days following infection most patients present with typical symptoms of gastrointestinal illness, such as nausea, vomiting and abdominal pain. As the infection worsens, bloody diarrhea develops due to intestinal lesions followed by swelling and hemorrhaging of the mesenteric lymph nodes (Baldari, Tonello et al. 2006). Although antibiotic treatment can be successful in cases of early diagnosis, mortality has been reported to exceed 50% (Baldari, Tonello et al. 2006).

#### 1.1.3.3 Inhalational anthrax

Inhalational anthrax is the rarest form of anthrax disease as well as the most lethal. One reason for the rarity is that anthrax spores tend to flocculate and fall to the ground similar to snowflakes, making inhalation of the spores difficult (Baldari, Tonello et al. 2006). The recently weaponized forms of anthrax spores are more easily transmitted as they are coated with charged molecules making the aerosol form more dispersible (Baldari, Tonello et al. 2006). Upon inhalation, spores are taken up by macrophages or dendritic cells in the lungs and brought to the lymph nodes were germination occurs. This first phase of illness involves non-specific symptoms such as malaise, fever and mild cough and can be difficult to diagnose. The second phase begins as the bacteria enter the lymphatic and blood systems resulting in bacteremia and toxemia. Symptoms associated with the second phase include: enlarged lymph nodes,

pulmonary edema with labored respiration and severe respiratory distress (Bhatnagar and Batra 2001; Baldari, Tonello et al. 2006). As the time course of the second phase is so rapid, antibiotic treatment is not effective and death occurs shortly after due to respiratory failure, sepsis and shock (Bhatnagar and Batra 2001; Baldari, Tonello et al. 2006).

#### 1.1.3.4 Treatment of Anthrax Disease

As mentioned previously, all forms of anthrax disease can be treated with antibiotics if diagnosed early while the bacteria are still multiplying and has not achieved colonization. It is also believed that during initial stages antibiotic treatment may inhibit the protein or RNA synthesis necessary for toxin production thus preventing toxic effects (Sherer, Li et al. 2007). When diagnosis is delayed antibiotic treatment is generally ineffective due to the high levels of toxin secreted by this time. In severe cases, which have progressed to sepsis and shock, vasopressors and corticosteroid therapy have been used to treat these symptoms (Sherer, Li et al. 2007). Current research has been focussed on developing new agents which can inhibit the activity of the anthrax toxins (Sherer, Li et al. 2007).

Vaccination would be the ideal method of preventing lethal cases of anthrax disease but is not currently a viable option for the general public. The first vaccine for *B. anthracis* consisting of heat-attenuated cultures was developed by Pasteur and was used to prevent anthrax disease in cattle and sheep. Over time the Pasteur vaccine was replaced by newer vaccines such as the Sterne live spore vaccine of which a derivative is still used for the vaccination of livestock (Bhatnagar and Batra 2001). Three vaccines are currently commercially produced for human use in Russia, the UK and the USA. The Russian vaccine is a derivative of the Sterne vaccine and is known to have several negative side effects, while the UK vaccine is an alum-precipitated

culture filtrate of the Sterne strain (Bhatnagar and Batra 2001). The USA produced Anthrax Vaccine Adsorbed (AVA) is made up of protective antigen produced by a nonencapsulated strain of *B. anthracis* (Bhatnagar and Batra 2001). All of these vaccines are now considered unacceptable as they do not provide protection against all natural strains and are associated with negative side effects.

#### 1.1.3.5 Virulence Factors

In all forms of anthrax disease, the bacteria require the suppression of the immune system in order to achieve colonization, but also rely on phagocytes to be transported to the regional lymph nodes (Tournier, Rossi Paccani et al. 2009). This is achieved by the two major virulence factors of *B. anthracis*; the anthrax capsule and the three component toxin. *B. anthracis* enters the body in the form of a spore, which is phagocytized by macrophages and dendritic cells and brought to the primary target, the lymph nodes. Once in the lymph nodes the spores germinate and the vegetative bacteria produce a capsule composed of poly-D-glutamic acid that protects the bacteria from phagocytosis and bactericidal molecules. This capsule has been found to be very important in the severity of anthrax disease, with strains lacking capsules being avirulent (Bhatnagar and Batra 2001). Vegetative bacteria also produce the three anthrax proteins, lethal factor (LF), edema factor (EF) and protective antigen (PA), which together suppress the immune system via activity against cytoplasmic targets. Both capsule and toxin production are dependent on temperature and dissolved bicarbonate. The optimal culture conditions for capsule and toxin synthesis are a temperature of 37°C and a defined media containing glucose and bicarbonate (Koehler 2009). These conditions are also found in mammalian bodies, with bicarbonate concentrations of 15-40 mM and a temperature of 37°C (Koehler 2009).

### 1.1.4 Anthrax Toxins

Like many other toxins acting on cytoplasmic targets, the anthrax proteins are A-B type toxins. A-B type toxins are composed of two functionally distinct components and can be different domains of a single polypeptide, or as in the case of the anthrax toxins, two separate proteins (Hicks, Hartell et al. 2005). The A portion has enzymatic activity on a cytoplasmic target while the B component functions by binding to a cellular receptor and assisting the A portion in reaching the cytoplasmic target. In the case of the anthrax toxins, lethal factor and edema factor are alternating A components and protective antigen functions as the B portion.

#### 1.1.4.1 History of anthrax toxins

The anthrax toxins were first discovered by in 1954 (Smith and Keppie 1954). As with the discovery of most toxins, bacteria free filtrates from infected animals or cultures of *B. anthracis* were used to inject laboratory animals. These animal studies indicated the sterile filtrates were lethal when injected and caused edema when injected intradermally. This led to the purification of three separate proteins which were not toxic individually. Factor II and factor III when injected into animals together were found to be lethal whereas factor II and factor I together caused edema when injected intradermally (Bhatnagar and Batra 2001). These studies led to the current naming of lethal factor (LF, factor III), edema factor (EF, factor I) and protective antigen (PA, factor II), with LF and PA together known as the lethal toxin and EF and PA the edema toxin.

#### 1.1.4.2 Genetic and Molecular Regulation of Toxins

The two major virulence factors of *B. anthracis*, the three component toxin and poly-D-glutamic acid capsule, are encoded on plasmids that were discovered in the 1980s. These

virulence plasmids are all that distinguish *B. anthracis* from the rest of the *B. cereus* group. A 96 kb plasmid, pX02, contains the *capBCADE* operon which encodes all the proteins necessary for capsule synthesis, assembly and transport, as well as the cap operon regulatory genes, *acpA* and *acpB* (Koehler 2009). All three toxins are encoded in a pathogenicity island on pXO1, a 182kb plasmid with 203 potential open reading frames (ORFs). The pathogenicity island contains the PA, LF and EF structural genes, *pagA, lef,* and *cya* respectively, as well as the regulatory gene *atxA* (anthrax toxin activator), germination operon *gerX* and an element resembling a class II cointegrative transposon (Koehler 2009). This element contains genes for transposase and a site specific recombinase.

Under optimal growth conditions, the PA structural gene (*pagA*) is highly expressed, with mRNA levels four fold higher than *lef* and 14 fold higher than *cya* (Bhatnagar and Batra 2001). Studies have shown that *in vitro* toxin production yields 20 mg/L PA, 5 mg/L LF and 1 mg/L EF (Bhatnagar and Batra 2001; Moayeri and Leppla 2009). The bacterial protein, anthrax toxin activator (AtxA) acts as a global transcription regulator for *B. anthracis* and has a strong positive effect on the toxin genes and capsule operon. The exact mechanism of regulation remains unclear, but mouse mutants lacking *atxA* gene are found to be highly attenuated for virulence (Bhatnagar and Batra 2001; Koehler 2009). Studies have indicated AtxA may have DNA binding activity, but binding at target gene promoters has not been observed, leading to the theory that DNA topology of promoter regions may play a role in AtxA regulation (Koehler 2009). As the presence of $CO_2$/bicarbonate has been found necessary for optimal toxin and capsule production, it is also believed that $CO_2$ plays a role in AtxA induced transcription of the toxin genes (Bhatnagar and Batra 2001).

### 1.1.4.3 Protective Antigen: Getting into the cell

Protective antigen is the B portion of the anthrax toxins, and is responsible for cellular binding and uptake of LF and EF into the cells. It is long flat 83-kDa protein consisting of 4 domains (Figure 1.1.2). The N-terminal domain 1 is a β sandwich with 4 small helices which interacts with 2 calcium ions to stabilize the structure (Young and Collier 2007). As seen in Figure 1.1.2 this domain contains two subdomains which are separated by furin cleavage, releasing domain 1a and generating the active $PA_{63}$. Domain 2 has a β-barrel core and contains the flexible 2β2-2β3 loop that acts as the transmembrane pore in the active heptameric PA. Domain 3 contains a ferridoxin-like fold and is believed to assist with self-association of $PA_{63}$. Domain 4 is a β-sandwich with immunoglobulin folds and is responsible for binding the cellular receptors (Young and Collier 2007; Collier 2009).

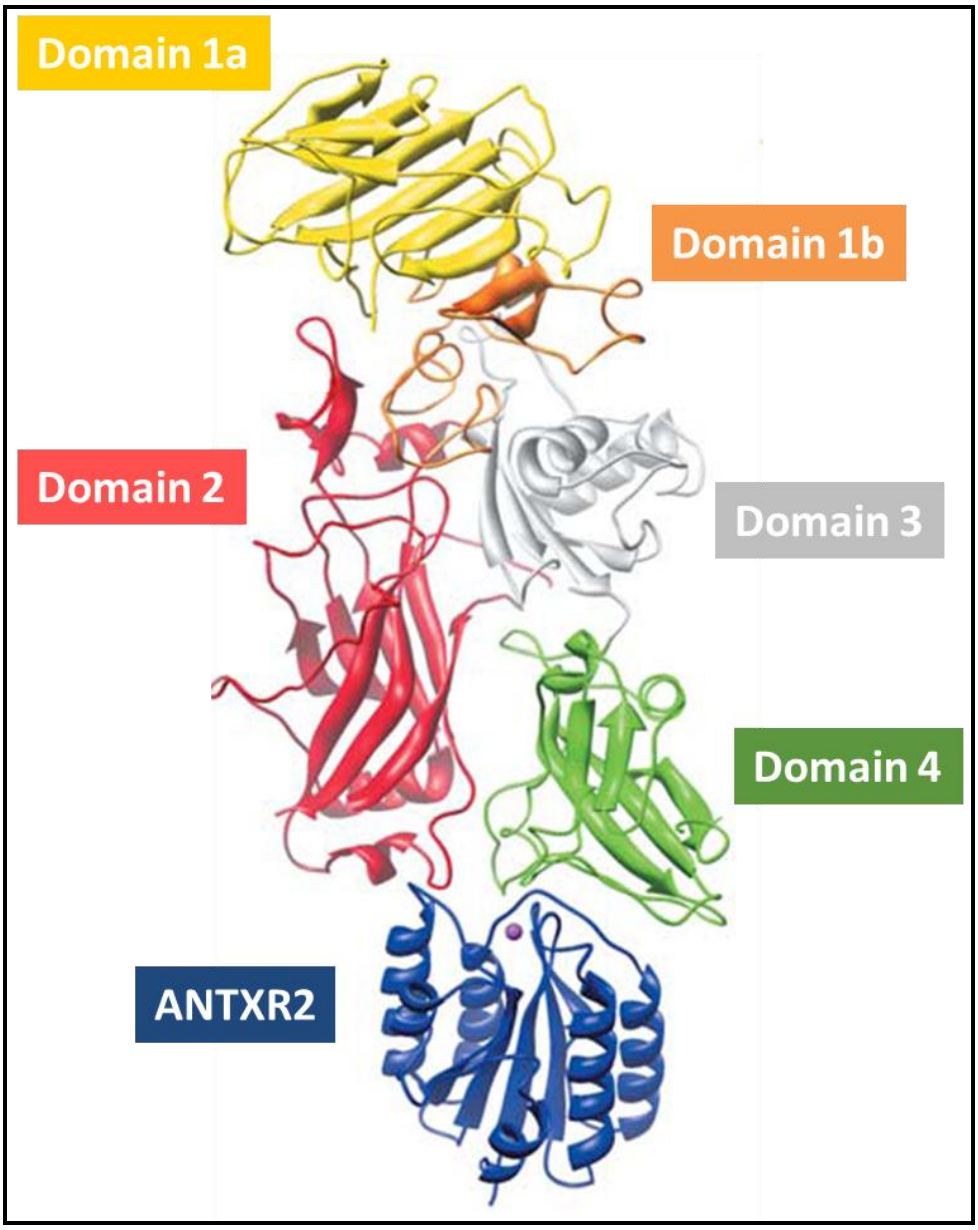


Figure 1.1.2: Protective antigen structure.

Monomeric PA bound to anthrax receptor 2 domain 1, the divalent cation is shown as a purple ball. Reprinted with permission from Nature Publishing Group: Nature (Santelli, Bankston et al. 2004).

The first step in toxin activity is the binding of full length $PA_{83}$ to cellular receptors. Two receptors have been identified which bind to PA: tumour endothelial marker-8 (ANTXR1) and capillary morphogenesis protein 2 (ANTXR2) (Young and Collier 2007; van der Goot and Young 2009). The two receptors are closely related and universally expressed, making all cell types susceptible to the toxins. Both receptors are transmembrane proteins with a single membrane spanning domain. The extracellular domain responsible for binding of PA is closely related to von Willebrand factor type A (VWA) domains (van der Goot and Young 2009). The VWA like domains contain metal ion-dependent adhesion site (MIDAS) which binds a divalent cation necessary for the binding of PA. Studies have shown ANTXR2 has a 1000 fold higher affinity for binding PA in comparison to ANTXR1 (van der Goot and Young 2009). A possible reason for this is a portion of buried protein at the binding interface of ANTXR2. The VWA domain of ANTXR2 interacts with PA domain 4 as well as residues in the receptor β4-α4 loop interact with PA domain 2 (van der Goot and Young 2009). Corresponding residues of ANTXR1 are different and do not make this extra stabilizing interaction with PA domain 2.

Figure 1.1.3 summarizes the activities of the anthrax toxin beginning with $PA_{83}$ binding to the cellular receptor. After receptor binding, the N terminal region of $PA_{83}$ is cleaved by a furin-like enzyme resulting in $PA_{63}$. This step is necessary as the N terminal region sterically inhibits the next step of oligomerization. The two calcium ions of domain 1 help to stabilize the PA-receptor complex, facilitating self-association of seven PA-receptor complexes which forms the heptameric prepore (Young and Collier 2007). Upon formation of the prepore domains 1 and 2 are on the inside of the ring while domains 3 and 4 are on the outside (Young and Collier 2007). The LF/EF binding site is located at the intersection of 2 PA molecules where a large hydrophobic surface of domain 1 is located. Due to the location of the binding site, LF and EF

binding can only occur once oligomerization has taken place. LF and EF compete for binding and the heptamer can bind 3 molecules under ligand saturating conditions (Young and Collier 2007).

Endocytosis of toxin-receptor complexes is ligand induced in order to prevent unproductive endocytosis. Endocytosis of $PA_{83}$ or monomeric $PA_{63}$ would result in LF and EF being left on the outside of the cell (van der Goot and Young 2009). Oligomerization and toxin binding causes the toxin-receptor complexes to cluster in detergent resistant membrane microdomains, also called lipid rafts. The formation of lipid rafts is necessary for internalization as it allows the receptor to interact with E3 ubiquitin ligase Cbl which leads to the ubiquitination of the receptor at a conserved lysine residue, triggering endocytosis (Young and Collier 2007; van der Goot and Young 2009). Ubiquitination is a common signal used to trigger endocytosis as well as protein sorting along the endocytic pathway (Mukhopadhyay and Riezman 2007). Endocytosis of the toxin-receptor complex is believed to occur via clathrin-coated pits, although clathrin-independent toxin uptake has been observed (Young and Collier 2007).

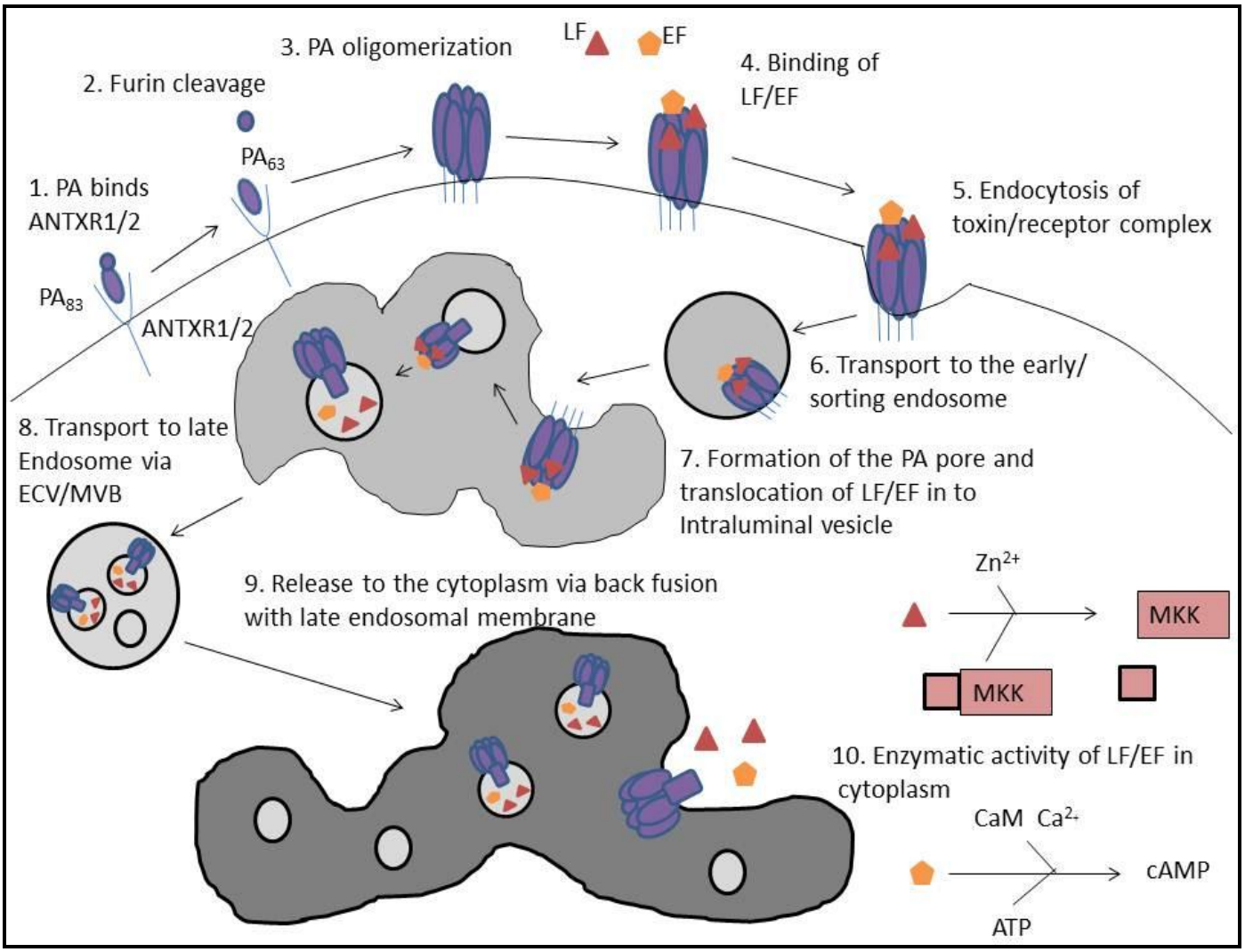


Figure 1.1.3: Summary of anthrax toxin activity.

Adapted from Young and Collier 2007, van der Goot and Young 2009, Gruenberg and van der Goot 2006 and Abrami *et al* 2004.

Studies have shown (Young and Collier 2007) that a cell surface protein, lipoprotein-receptor-related protein 6 (LRP6), may act as a co-receptor and stimulate internalization. LRP6 is a type 1 membrane protein that is part of the Wnt-signaling pathway and is believed to interact with toxin receptors and undergo tyrosine phosphorylation (Young and Collier 2007; van der Goot and Young 2009). The exact function in toxin endocytosis remains controversial, although LPR6 antibodies were found to interfere with anthrax intoxication (Young and Collier 2007). ARAP3, a phosphoinositide-binding protein with GTPase activating domains for ARF6 and Rho GTPases, also plays a potential role in toxin internalization. ARF6 and Rho GTPases play roles in membrane trafficking and cytoskeletal actin dynamics which may promote toxin uptake (van der Goot and Young 2009). ARAP3 also interacts with CIN85 which then interacts with E3 ubiquitin ligase Cbl, potentially recruiting Cbl to the receptor which is necessary for ubiquitination to trigger endocytosis (van der Goot and Young 2009).

After endocytosis the toxin-receptor complex follows the endocytic pathway beginning with transport to the early endosome where protein sorting occurs. Like many pathogens, *B. anthracis* has evolved mechanisms to utilize the endocytic pathway in order to gain entry to the cell and access to cellular targets (Gruenberg and van der Goot 2006). The endocytic pathway can lead to lysosomes and could result in the anthrax toxins being degraded prior to reaching their cellular targets, but *B. anthracis* has developed means to prevent this. Once in the early endosome, the toxin complex is sorted to the vesicular region where the acidic pH of the lumen triggers the PA heptamer to insert into the membrane of intraluminal vesicles and form the PA pore.

Studies have shown that the PA heptamer preferentially inserts into the intraluminal membrane and not the endosomal membrane (Abrami, Lindsay et al. 2004). The lower pH of the lumen allows the PA heptamer to undergo conformational changes which enable it to insert into the membrane. Negative stain EM images of the pore indicate that it has a mushroom shape with a 125Å diameter cap and 100Å long stem (Collier 2009). The top of the cap consists of the seven copies of domain 3 which are seen as radiating knobs, while the 2β2-2β3 loop of domain 2 makes up the membrane spanning region. It is the 2β2-2β3 loops of domain 2 that undergo the major conformational changes to form at 14 strand β-barrel through which EF and/or LF will translocate through (Young and Collier 2007). Differences in the pH threshold for pore formation have been found depending on the specific receptor to which the toxin is bound. ANTXR1 has an optimum threshold of pH 6.2 while ANTXR2 has a threshold of pH 5.2 (Young and Collier 2007; van der Goot and Young 2009). This pH difference is thought to be due to differences in the interaction between the receptor and domain 2 of PA, and may cause pore formation when bound to ANTXR2 to occur in later endosomal compartments where the pH is higher (Young and Collier 2007; van der Goot and Young 2009).

The low pH of the endosome also triggers the partial unfolding and entry of the N-terminus of LF/EF into the PA pore. The pore is predicted to be 15Å, and at this size it would be necessary for larger proteins to unfold prior to entering the pore (Young and Collier 2007). Entry into the pore is dependent on the protein having a net positive charge. The N-terminus of both LF and EF contain an equal number of positive and negative charged amino acid residues, the acidic pH of the endosome causes neutralization of the basic residues resulting in a net positive charge and entry of the protein into the negatively charged pore via electrostatic forces (Collier 2009). The conserved phenylalanine of PA domain 2 (F427) is essential for

translocation. The seven neighbouring F427 residues have been termed the phenylalanine clamp (Phe clamp) and are located 15-20Å apart forming a narrow constriction in the lumen of the pore (Collier 2009). Upon entering the pore, the N-terminus of LF/EF interacts with this Phe clamp creating a seal around the translocating protein which blocks ion flow and maintains the pH gradient necessary for translocation (Young and Collier 2007; Collier 2009). The pH gradient between the *cis* (endosomal lumen) and *trans* (lumen of intraluminal vesicle) drives translocation via a charge state Brownian ratchet mechanism (Figure 1.1.4). As the pore is cationic selective, anionic portions of the protein are protonated and diffuse through the pore where the higher pH results in deprotonation preventing back diffusion (Young and Collier 2007; Collier 2009).

After translocation through the PA pore, the toxins are located in the lumen of intraluminal vesicles, far away from their cytoplasmic targets but protected from degradation during the next part of the journey. Intraluminal vesicles containing the toxins are packaged in multivesicular bodies (MVB) also termed endosomal carrier vesicles (ECV), and trafficked to late endosomes in a microtubule dependent manner (Abrami, Lindsay et al. 2004; Gruenberg and van der Goot 2006; Young and Collier 2007; van der Goot and Young 2009). After reaching the late endosome, the toxins are released to the cytoplasm by back diffusion with the limiting membrane. It is unknown why the toxins are delivered to the late endosome and not released at the early endosome; one theory suggests this method delivers the toxin in closer proximity to cellular targets (Abrami, Lindsay et al. 2004).

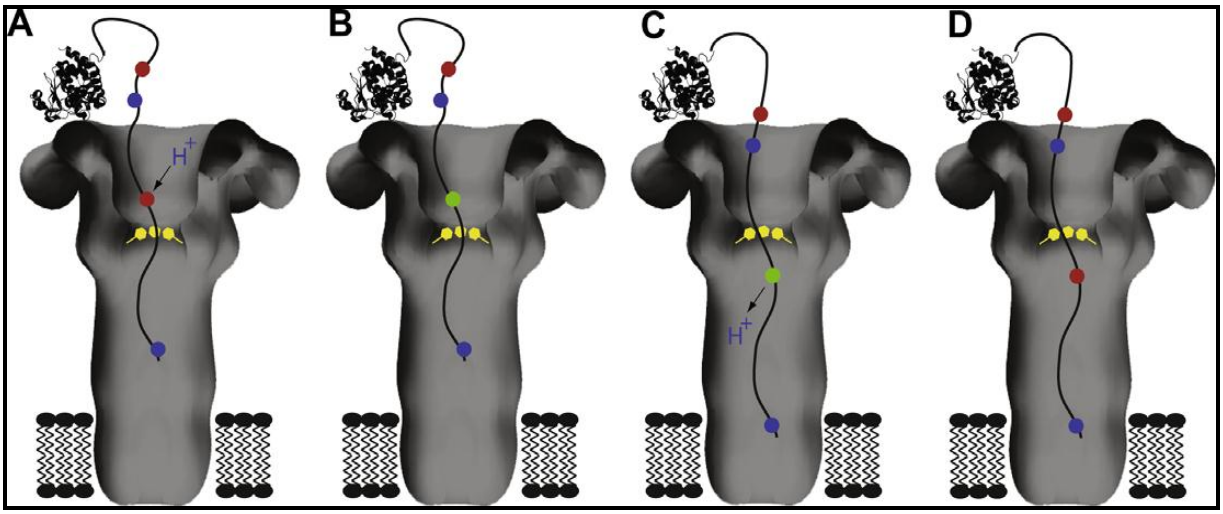


Figure 1.1.4: Mechanism of translocation through the PA pore.

(A) The partially unfolded LF enters the pore through the Phe clamp (yellow) where an anionic portion (red) cannot diffuse through the pore until it is protonated. (B) The now protonated portion (green) can now pass through the pore where it is then deprotonated (C) preventing back diffusion (D).

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### 1.1.4.4 Edema Factor: Structure and Enzymatic Activity:

Edema factor is one of the A components of the anthrax binary toxin and functions as an adenylyl cyclase, resulting in elevated intracellular cyclic AMP (cAMP) levels. EF remains an inactive enzyme until it is activated by the eukaryotic calcium sensor calmodulin (CaM). The 89-kDa EF has 3 modular domains; the N-terminal PA binding domain (PABD), the adenylyl cyclase (AC) domain consisting of two subdomains ($C_A$ and $C_B$), and a 17-kDa helical domain that plays a role in keeping EF in an inactive state when CaM is absent (Figure 1.1.5). The catalytic site of the AC domain is located at the interface of the two subdomains and has been shown to be a disordered loop in the inactive state (Tang and Guo 2009). Calmodulin activates EF by first binding to the EF helical domain and then the C-terminus of CaM is inserted between the catalytic core and helical domain of EF (Figure 1.1.5). This insertion results in a conformational change that stabilizes the catalytic core (Tang and Guo 2009). The high AC activity of EF rapidly converts ATP into cAMP, elevating intracellular levels of this important secondary messenger. Cyclic AMP regulates diverse cellular responses via binding of three families of signal transducers: cAMP dependant protein kinases, EPAC (guanine nucleotide exchange factor for Ras GTPase homologs Rap 1 and Rap2) and cyclic nucleotide-gated channels (Tang and Guo 2009). These signal transducers can have wide ranging effects on important cellular activities such as: metabolism, cell differentiation, proliferation, vesicle trafficking and ion transport.

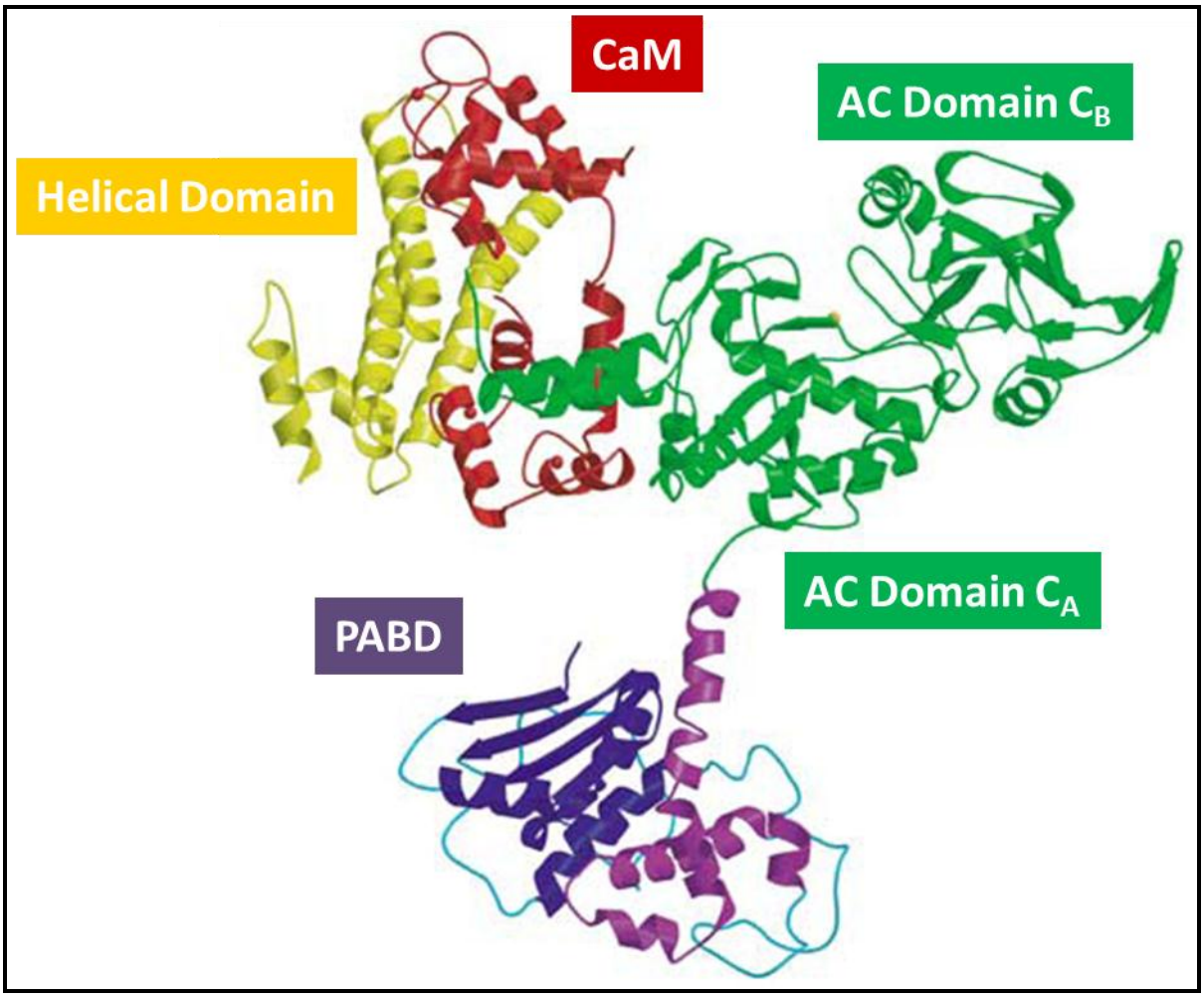


Figure 1.1.5: Edema Factor structure in complex with CaM, indicating the 3 domains.

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### 1.1.4.5 Lethal Factor: Structure and Enzymatic Activity:

Lethal factor is the alternate A component of the anthrax toxins and is a zinc-metalloprotease that cleaves mitogen-activated protein kinase-kinases (MAPKKs). Lethal factor is a 90-kDa protein consisting of 4 domains (Figure 1.1.6) with a structure similar to botulism neurotoxin. The major function of domain 1 is binding to PA, as such it has high structural similarity to the PA binding domain of EF (Tonello and Montecucco 2009). Domain 1 consists of 4 stranded and 2 stranded β sheets packed with a bundle of 9 helices. Domain 2 is similar in structure to the catalytic portion of VIP2 ADP ribosyltransferase of *Bacillus cereus* and has been found to be important for binding of MAPKK. Domain 3 forms the long cleft necessary for recognition and binding of MAPKK. Domain 4 is the enzymatic portion of the protein and is composed of nine helical bundles packed against a 4 stranded β sheet. This domain contains the HExxH fingerprint of a zinc-metalloprotease (Tonello and Montecucco 2009).

The substrate of LF, MAPKK, is in the middle of an important three component phosphorylation cascade that is activated by a wide range of extracellular stimuli and is responsible for mediating a large number of responses. Extracellular stimulation leads to activation of mitogen-activated protein kinase kinase kinases (MKKKs) which phosphorylate MAPKKs, which then phosphorylate mitogen-activated protein kinases (MAPKs) (Turk 2007). MAPKs are responsible for activating a number of signaling pathways such as the ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 pathways, which play important roles in cell proliferation, cell cycle regulation, immune modulation and survival against toxic insults (Moayeri and Leppla 2009). LF cleaves a specific site of the N-terminus of MAPKKs. Although outside of the catalytic site, the N-terminus contains the MAPK docking

motif, or D site (Turk 2007). The D site is necessary for forming complexes with MAPK and cleavage at this site reduces binding affinity, preventing phosphorylation of MAPK and disrupting the signaling cascade.

#### 1.1.4.6 Lethal Factor and Edema Factor: Cellular and Systemic Effects:

The enzymatic activity of both EF and LF targets ubiquitous cell signaling pathways and therefore has extensive effects on a variety of cell types (Moayeri and Leppla 2009). Once the *B. anthracis* spores have reached their primary target (the lymph nodes), the spores germinate and begin secreting toxins which work synergistically to suppress both the innate and adaptive immune system, therefore allowing bacteria to multiply unchecked. EF and LF suppress the immune system through their enzymatic activities which manipulate the signaling cascades in a variety of immune cells as summarized in Figure 1.1.7.

Anthrax toxins target the phagocytic cells which function as the first line of defense in the innate immune response. By inhibiting the activity of macrophages, dendritic cells (DCs) and polymorphonuclear cells (PNMs), vegetative bacteria are able to achieve colonization and delay the host response. *B. anthracis* normally triggers a strong pro-inflammatory response by activating toll-like receptors (TLRs) on phagocytic cells, but once the anthrax toxins are released into the blood stream this response is inhibited. PNMs, phagocytes in the blood, are recruited via chemokine production to destroy pathogens. LF blocks PNM recruitment by inhibiting chemokine production of epithelial cells and dendritic cells (Tournier, Rossi Paccani et al. 2009). PNMs ability to kill pathogens is also disrupted as LF prevents generation of the bactericidal superoxide burst (Tournier, Rossi Paccani et al. 2009). LF further enables *B. anthracis* to escape the host immune response by preventing the antigen presenting activity of DCs.

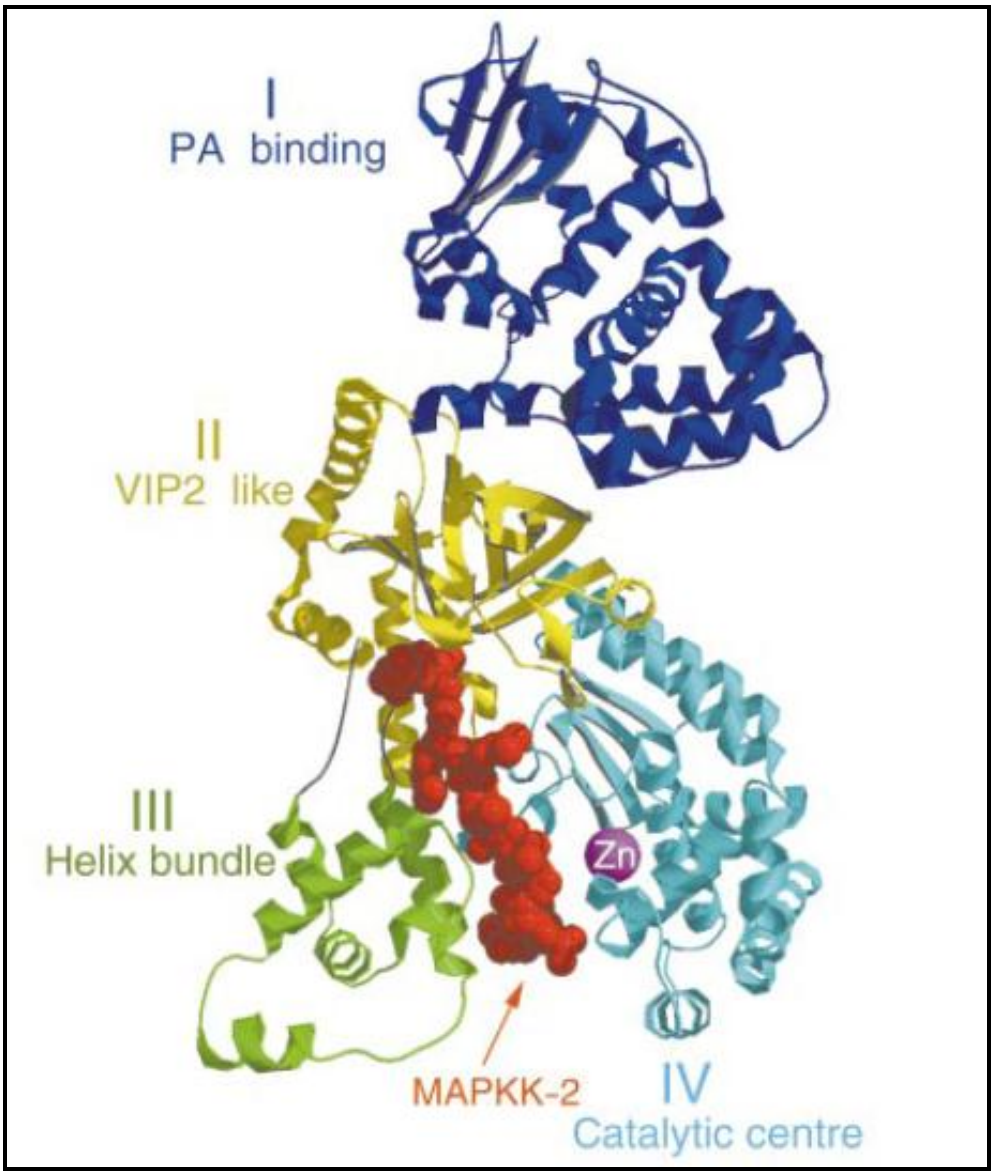


Figure 1.1.6: Lethal Factor structure indicating the 4 domains, bound to MAPKK substrate and $Zn^{2+}$ ion.

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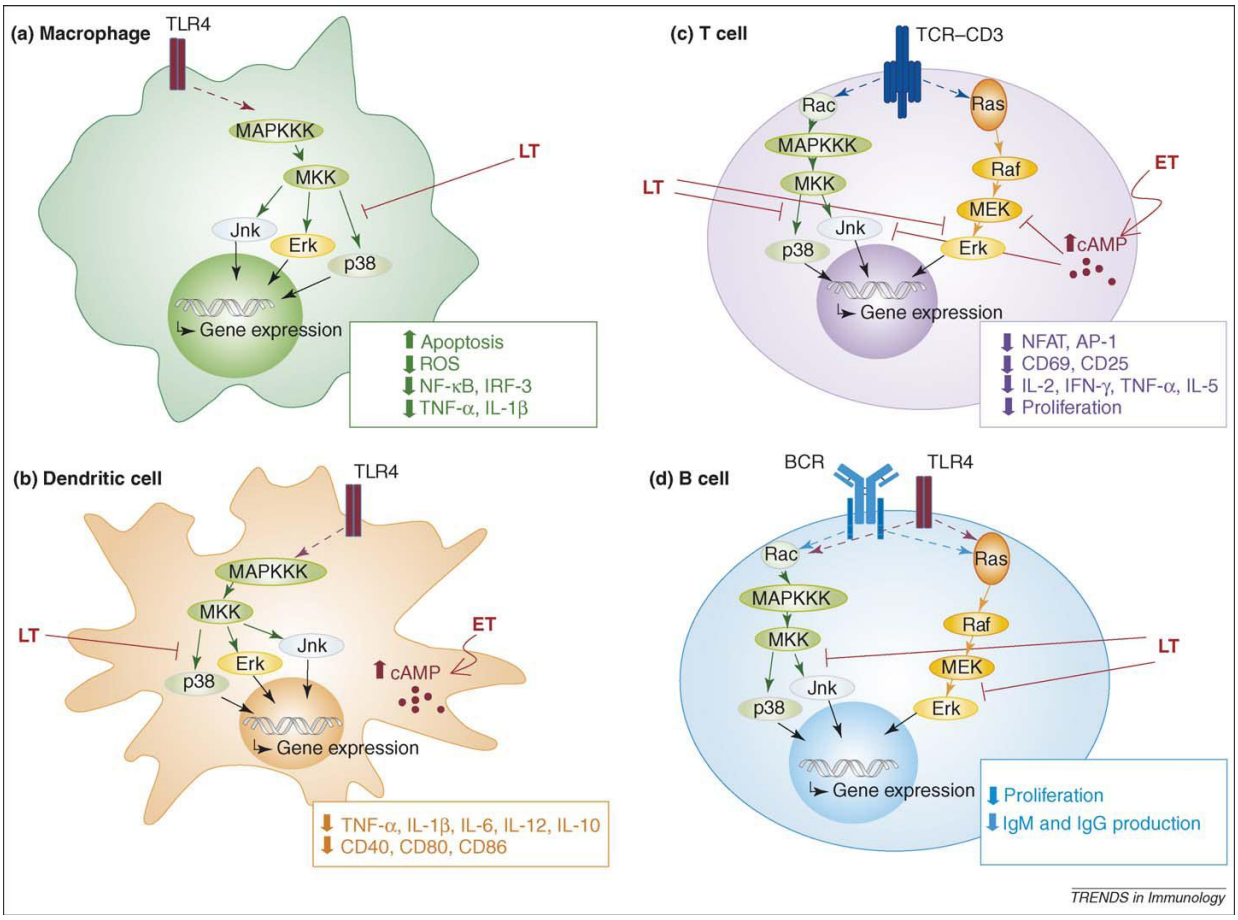


Figure 1.1.7: Summary of LF and EF effects on immune cells.

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Both LF and EF inhibit DC secretion of pro-inflammatory cytokines such as IL-6, TNF-α, IL-12 and IL-1β, which leads to impairment of T and B cell response (Baldari, Tonello et al. 2006; Tournier, Rossi Paccani et al. 2009). The anthrax toxins also prevent DCs from initiating the differentiation of T cells to T helper (Th1) cells, which are thought to be crucial for host survival against *B. anthracis* (Tournier, Rossi Paccani et al. 2009).

LF has two major effects on macrophages, the induction of apoptosis and suppression of pro-inflammatory cytokine expression. MAPK signaling cascades are required for macrophage activation, production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) and production of bactericidal enzymes (Baldari, Tonello et al. 2006). Elevation of cAMP levels also indirectly affects the pro-inflammatory response. One of the most studied effects of LF is the induction of apoptosis in macrophages. By cleaving upstream MAPKKs, LF prevents p38 and NF-κB activation thus disrupting the expression of pro-survival genes, leading to apoptosis (Baldari, Tonello et al. 2006).

By inhibiting the pro-inflammatory cytokine secretion of innate immune cells, anthrax toxins also prevent the maturation and activity of adaptive immune cells. PA has been shown to bind to T and B cells, indicating that anthrax toxins have direct effects on these cells as well (Tournier, Rossi Paccani et al. 2009). LF is a strong suppressor of T cell activation and proliferation by directly interrupting MAPK signaling cascades. EF also suppresses T cell activation and the two toxins act synergistically to prevent T cell activation even at low concentrations by interfering with T cell antigen receptor signaling (Tournier, Rossi Paccani et al. 2009). The anthrax toxins also prevent T cell chemotaxis by interfering with chemokine receptor signaling. Chemokine receptors control the trafficking and homing of T cells by

modulating AC activity and the tyrosine kinase pathway which activates the MAPK pathway. By increasing the cAMP concentration and cleaving MAPKKs the toxins obstruct this chemotactic signaling, thus hindering T cell migration to and from the lymph nodes (Tournier, Rossi Paccani et al. 2009). This disruption of T cell chemotaxis plays a role in the delay of bacterial clearance and wound healing (Tournier, Rossi Paccani et al. 2009). The MAPK signaling cascade is also important in B cell proliferation and antibody production and as such LF inhibits B cell activity (Tournier, Rossi Paccani et al. 2009).

By inhibiting both the innate and adaptive immune responses, *B. anthracis* is able to evade the host defenses and proliferate. In targeting such wide reaching signaling cascades the toxins are able to affect a wide variety of cells and activities. Targeting these complex signaling components also makes it difficult to determine relevant *in vivo* targets and fully comprehend exactly how the toxins contribute to the pathogenesis, symptoms and mortality associated with anthrax disease.

Most *in vivo* studies investigating the systemic effects of anthrax toxins have been conducted in rodents. Early rodent studies indicated that EF was less lethal than LF resulting in few studies further investigating systemic effects of EF until recently, when EF was observed to cause death at lower doses and was associated with a more rapid onset of symptoms than LF (Sherer, Li et al. 2007; Turk 2007). In mice, lethal doses of EF cause hemorrhaging lesions resulting in widespread tissue damage and multi-organ failure accompanied by hypotension, shock and loss of circulatory fluids (Sherer, Li et al. 2007; Turk 2007; Moayeri and Leppla 2009). It remains unclear exactly how the enzymatic activity of EF results in these symptoms. It is hypothesized that the EF produced increase in cAMP and subsequent activation of cAMP

dependent protein kinase could lead to arterial relaxation, extravasation of fluid and shock (Sherer, Li et al. 2007).

Observations from rodent studies indicate that LF induces extravasation of fluids, shock, hypoxia and atypical vascular collapse with an absence of cytokine involvement (Sherer, Li et al. 2007; Turk 2007; Moayeri and Leppla 2009). As with EF, the exact relationship between the enzymatic activity of LF and the vascular collapse and shock leading to death remains unclear. As the disruption of the MAPK and cAMP related signaling pathways leads to an overall suppression of the immune system, it is apparent that anthrax generated shock is not caused by excessive inflammatory response as in classic bacterial sepsis. Theories suggest toxin activity in endothelial cells contributes to shock. Interruption of the MAPK related pathways, specifically the ERK pathway, induces apoptosis of endothelial cells lining blood vessels as well as a decrease in barrier function of lung microvascular endothelial cells causing vascular leakage (Agrawal and Pulendran 2004; Sherer, Li et al. 2007). Although inhibiting the immune system is an important activity of the anthrax toxin in promoting infection, it appears to be the effect on non-immune cells that contributes to the lethality of the toxins.

The use of rodent models in studying toxin activity *in vivo* presents problems in relating these findings to anthrax disease in humans. The concentration of EF found to be lethal in mouse studies is not actually produced during human infections, causing scientists to question the relevance of doses used in rodent models. Only trace amounts of EF are detected in infected animals, with *in vivo* studies indicating LF and EF are produced in a ratio of 5:1 (Moayeri and Leppla 2009). As well differences in lethality of LF have been found in different rat and mouse strains. Sensitive strains die as quickly as 37 minutes when a saturating dose of lethal toxin is

administered intravenously, while some strains can be resistant to relatively high doses of toxin or can take days to succumb to the toxin (Moayeri and Leppla 2009). This wide range of lethality cannot be solely attributed to differences in anthrax toxin receptor expression, alluding to other differences in genetic makeup most likely with multiple genetic loci involved (Moayeri and Leppla 2009). The sensitivity of macrophages to lethal toxin induced rapid lysis appears to play a role in rodent susceptibility to death. Mice were found to have intermediate levels of sensitivity suggesting multi loci are responsible, while rats either succumb to low levels of toxin or are completely resistant implying this trait is controlled by only one locus (Moayeri and Leppla 2009).

The discovery of LF induced rapid lysis of mouse macrophages has led to much study in this area as well as controversy over the relevancy to human anthrax disease. Human macrophages do not undergo this rapid lysis and so far no definitive link has been made between LF cleavage of MAPKK and mouse macrophage lysis (Turk 2007; Moayeri and Leppla 2009). The rapid death caused by LF is characteristic of necrosis and signified by univalent cation permeability, intracellular ATP depletion, loss of mitochondrial function, termination of protein synthesis, loss of plasma membrane integrity and ultimately cell lysis (Turk 2007). Resistant rodent macrophages, as with other several other cell types including human macrophages, can also undergo slower apoptotic death via interruption of MAPK signaling pathways as mentioned previously.

Sensitivity of mouse macrophage rapid lysis has been mapped to the extremely polymorphic *Nalp1b* gene in the *Ltxs1* locus on chromosome 11 (Boyden and Dietrich 2006). Five polymorphic alleles have been identified with alleles 1 and 5 conferring sensitivity in a

dominant manner (Boyden and Dietrich 2006). *Nalp1b* encodes a member of the nod-like receptor (NLR) protein family which is homologous to the human Nalp1. NLR proteins are intracellular inflammatory mediators and function similar to the membrane spanning TLRs by sensing "danger" signals and activating pro-inflammatory signaling (Turk 2007). Nalp1b contains a CARD (caspase activation and recruitment domain) and is required for recruitment of caspase-1 and formation of the multi-protein inflammasome complex which activates an immune response and cell death (Turk 2007; Moayeri and Leppla 2009). LF induces this caspase-1 activation and rapid lysis only in sensitive macrophages, but it is unclear how the toxin is involved and there is no direct link of MAPKK cleavage to Nalp1b activation. It is possible that LF directly cleaves Nalp1b leading to activation or there may be an unidentified LF substrate that acts as an upstream activator or inhibitor of Nalp1b (Boyden and Dietrich 2006; Turk 2007).

## 1.2 Ricin Toxin

### 1.2.1 Introduction to *Ricinus communis*: the castor oil plant

Unlike the bacterial anthrax toxins, ricin is a plant protein synthesized by *Ricinus communis*, the castor oil plant. This flowering plant is a member of the spurge family (Euphorbiaceae), which originated in Asia and Africa and can now be found in all temperate and subtropical regions (Doan 2004; Olsnes 2004; Spivak and Hendrickson 2005). There are several different varieties, or cultivars, of *R. communis* plants each having slight differences in the appearance of the plant and seeds. Plants are generally shrub like but can grow to the size of a small tree in warmer climates. The palm like leaves range in colour from green to more dramatic purples and reddish browns depending on the variety. Castor oil plants produce a fruit which is a

greenish capsule containing oblong glossy light brown bean-like seeds covered in black, brown or white spots (Doan 2004; Audi, Belson et al. 2005).

Castor seeds have been used since ancient times in folk medicine, with the oil extracted from these seeds being described in classical Egyptian and Greek medicine as a laxative and purgative (Bradberry, Dickers et al. 2003; Doan 2004; Olsnes 2004). The oil was also used in ancient times for fueling lamps as it was readily available and burned relatively slow. In modern times castor oil is more commonly used for its lubricating qualities. Modern hot pressing of seeds followed by solvent extraction of the oil produces specialist oils and lubricants used in paints, varnishes, jet engines and industrial machinery (Bradberry, Dickers et al. 2003; Doan 2004; Audi, Belson et al. 2005). Castor plants are grown commercially with a global seed production of one million tons per year (Sehgal, Khan et al. 2010).

Ricin, a highly toxic protein, is produced in the endosperm cells of castor seeds and can be easily isolated during castor oil extraction. The seed pulp waste that remains following castor oil extraction contains approximately 1-5% ricin by weight (Audi, Belson et al. 2005). The toxin is easily purified using chromatographic methods producing a white powder which is soluble in water and stable over a wide pH range. Ricin is heat resistant and requires heating at 80°C for 10 minutes or at 50°C for one hour for inactivation (Bradberry, Dickers et al. 2003).

Different isoforms of ricin can exist depending on the variety of seeds, the level of seed maturation and cultivation conditions. Early studies found two different isoforms, ricin D and ricin E along with the closely related weakly cytotoxic *Ricinus communis* agglutinin (RCA). Ricin D was reported to be produced by large grain seeds, while small grain seeds produced both ricin D and E, although it is now believed the appearance of seeds is not always a reliable

indicator of toxin isoform (Sehgal, Khan et al. 2010; Sehgal, Kumar et al. 2011). Ricin D and E have been found to have slight differences in isoelectric points, amino acid sequence as well as cytotoxicity due to difference in the ability to bind saccharides. More recent studies (Sehgal, Khan et al. 2010; Sehgal, Kumar et al. 2011) have isolated 3 different isoforms termed ricin I, II and III each varying in the degree of glycosylation and cytotoxicity. Ricin isoform III was found to be the most highly glycosylated and to induce the greatest level of cytotoxicity. The researchers found the higher cytotoxicity to be caused by the greater glycosylation which provided more stability to the protein allowing stronger binding to cell surface receptors (Sehgal, Kumar et al. 2011).

### 1.2.2 Ricin Poisoning

All forms of ricin poisoning generally begin with non-specific symptoms, while further symptoms and severity depend on the route of exposure and dose received. As ricin is not selective for a specific cell type, the toxin can affect all cell types and is toxic via all routes (Bradberry, Dickers et al. 2003; Bigalke and Rummel 2005), although most cases result from ingestion, inhalation or injection of ricin. Diagnosis of ricin poisoning can be difficult as symptoms are similar to gastroenteritis or respiratory illness. Clues that may indicate ricin poisoning include an unexpected progression of symptoms and a credible evidence of ricin exposure (Audi, Belson et al. 2005). Ricin is most commonly detected in blood and bodily fluids by radioimmunoassay and ELISA (Doan 2004). As ricin poisoning is caused by exposure to a toxic protein rather than infection by a microorganism, person to person transmission does not occur.

#### 1.2.2.1 Ingestion

Human cases of ricin ingestion are general due to the consumption of castor seeds (rather than purified ricin) and although this route of exposure is common it is thought to be the least toxic (Bradberry, Dickers et al. 2003; Spivak and Hendrickson 2005). Significantly higher doses of ricin are necessary to cause illness when ingested in comparison to other modes of exposure. The $LD_{50}$ in mice for ingested ricin is approximately 30mg/kg while the lethal dose in humans is estimated to be 1-20 mg/kg or approximately 8 seeds (Audi, Belson et al. 2005; Musshoff and Madea 2009). The lower toxicity for ingested ricin is mostly due to poor gastrointestinal absorption of the toxin and the limited release of the toxin from the castor seed during eating (Bradberry, Dickers et al. 2003; Spivak and Hendrickson 2005). As chewing facilitates the release of ricin from the seed, the degree of seed mastication greatly influences the severity and outcome of poisoning (Bradberry, Dickers et al. 2003). Onset of nonspecific symptoms, such as nausea, vomiting, diarrhea and abdominal pain can be delayed for up to 12 hours post ingestion (Audi, Belson et al. 2005). In cases involving higher doses of ricin symptoms may further progress to hypotension, liver failure, and renal dysfunction eventually leading to death from multi-organ failure or cardiovascular collapse (Audi, Belson et al. 2005).

#### 1.2.2.2 Injection

Ricin poisoning by injection is much more lethal although less common than exposure by ingestion. Few cases of human exposure via injection have been recorded (Audi, Belson et al. 2005). This mode of exposure has been most notoriously used in cases of assassination and accidental poisoning by this means is unlikely. There is little published on human injection of ricin but the $LD_{50}$ for mice is approximately 5-10 µg/kg (Audi, Belson et al. 2005; Musshoff and

Madea 2009). Initial symptoms can be delayed up to 10-12 hours post injection and include generalized weakness, fever, headache, dizziness, nausea, hypotension and abdominal pain (Audi, Belson et al. 2005). In severe cases death may occur due to multisystem organ failure.

#### 1.2.2.3 Inhalation

Inhalation is the most lethal route of ricin poisoning as well as the most likely means of exposure to be used in cases of bioterrorism. Lethality and severity of symptoms is greatly influenced by particle size, as smaller particles are able to deposit deeper in the respiratory tract thus causing higher morbidity (Audi, Belson et al. 2005; Griffiths, Phillips et al. 2007; Musshoff and Madea 2009). Studies in mice have found an $LD_{50}$ of 3-5ug/kg when exposed to particles smaller than 5 µm (Audi, Belson et al. 2005; Musshoff and Madea 2009) with human lethal dose estimates at 5-10 µg/kg (Bradberry, Dickers et al. 2003). It is important to point out that rodents are obligate nasal breathers, while humans are not, and this could affect the particle deposition and therefore cause differences in toxicology between mice and humans (Griffiths, Phillips et al. 2007). As most studies on inhalation toxicology of ricin have been conducted on rodents more recent studies have focused on non-human primates. These studies have found lethal doses between 15 and 5.8 µg/kg in rhesus monkeys and African green monkeys (Griffiths, Phillips et al. 2007). Symptoms of poisoning are typical of respiratory illness and may begin within a few hours of inhalation or be delayed up to eight hours (Audi, Belson et al. 2005; Musshoff and Madea 2009). Early symptoms may include coughing, dyspnea, fever and tightness of chest and in severe cases will progress to respiratory distress and death.

#### 1.2.2.4 Treatment of Ricin Poisoning

There is no specific treatment for ricin poisoning and therapy is generally supportive and targeted to the specific symptoms. Treatment may include replacement of electrolytes and fluids to prevent dehydration from vomiting and diarrhea as well as artificial respiration and medication for seizures and low blood pressure (Doan 2004; Bigalke and Rummel 2005). In cases of ingestion treatment may include superactivated charcoal and purgatives in order to clear out the digestive track (Bigalke and Rummel 2005). Death from ricin poisoning generally occurs within three to five days of exposure, patients surviving longer normally recover.

Vaccination against ricin poisoning is not currently available for the general public as the risk of ricin exposure is low. Research is currently focused on the development of vaccines for at risk military personnel and prophalaxis treatment to be administered in the event of a bioterrorism related release of ricin. Animal studies have found active immunization and passive prophlaxis to be effective when administered within a few hours of exposure, except in cases of aerosolized ricin (Doan 2004). This is unfortunate as the most likely route of exposure to be used in bioterrorism related release of ricin is inhalation. Currently the most promising vaccine is a genetically engineered ricin A chain which lacks enzymatic activity but is able to elicit an antibody response (Doan 2004).

### 1.2.3 Ricin Toxin

#### 1.2.3.1 Structure and Biosynthesis:

Ricin is a type II ribosome inactivating protein (RIP) with glycosidase activity. The members of this family of proteins are closely related to the more common type I ribosome

inactivating proteins. Type I RIPs consist of a single enzymatic chain and are generally non-cytotoxic, while the structurally and functionally identical type II enzymatic chain is linked to an additional B chain that binds cell surface receptors causing cytotoxicity (Lord, Jolliffe et al. 2003). The ricin toxin is a 66-kDa globular glycosylated heterodimer, consisting of an A and B chain linked by a single disulfide bond (Figure 1.2.1A). Ricin toxin A (RTA) is the 32-kDa enzymatic chain and contains three domains arranged in eight α-helices and eight β-sheets, which form the pronounced cleft of the active site (Lord, Jolliffe et al. 2003; Musshoff and Madea 2009). As with other A-B type toxins, ricin toxin B (RTB) binds cellular receptors and gains access to the cytoplasmic target. RTB contains no α-helices or β-sheets, but is a 34-kDa bilobal structure with each of the two homologous lobes containing a sugar binding site (Lord, Jolliffe et al. 2003). It is believed that the B chain evolved from a series of gene duplications as each lobe consists of 3 galactose binding domains (α, β and γ) although only 1α and 2γ are functional (Olsnes 2004).

Ricin toxin is produced in fatty endosperm cells of mature castor seeds as a single polypeptide chain precursor known as proricin (Lord, Jolliffe et al. 2003; Bigalke and Rummel 2005; Lord, Roberts et al. 2005). Although proricin does contain functional sugar binding sites it is non-toxic as it lacks enzymatic activity. As seen in Figure 1.2.1B, proricin contains a 26 residue signal peptide and a nine residue propeptide at the N terminal end, as well as a 12 residue propeptide linking RTA and RTB all of which are absent in mature ricin. The signal peptide allows for translocation of proricin to the ER lumen where the peptide is removed and the protein undergoes glycosylation and disulfide bond formation (Lord, Jolliffe et al. 2003). The short linker peptide between RTA and RTB is a targeting signal that results in transportation of proricin to storage vacuoles via the secretory pathway (Lord, Roberts et al. 2005). Once inside

the vacuole mature ricin is generated by proteolysis of the N terminal and internal propeptides. It is through the generation of a non-toxic precursor that *R. communis* endosperms cells are able to synthesize and store large amounts of highly toxic protein (estimated to be up to 5% of total cellular particulate protein) with no damage to the endogenous ribosomes (Lord, Jolliffe et al. 2003; Lord, Roberts et al. 2005).

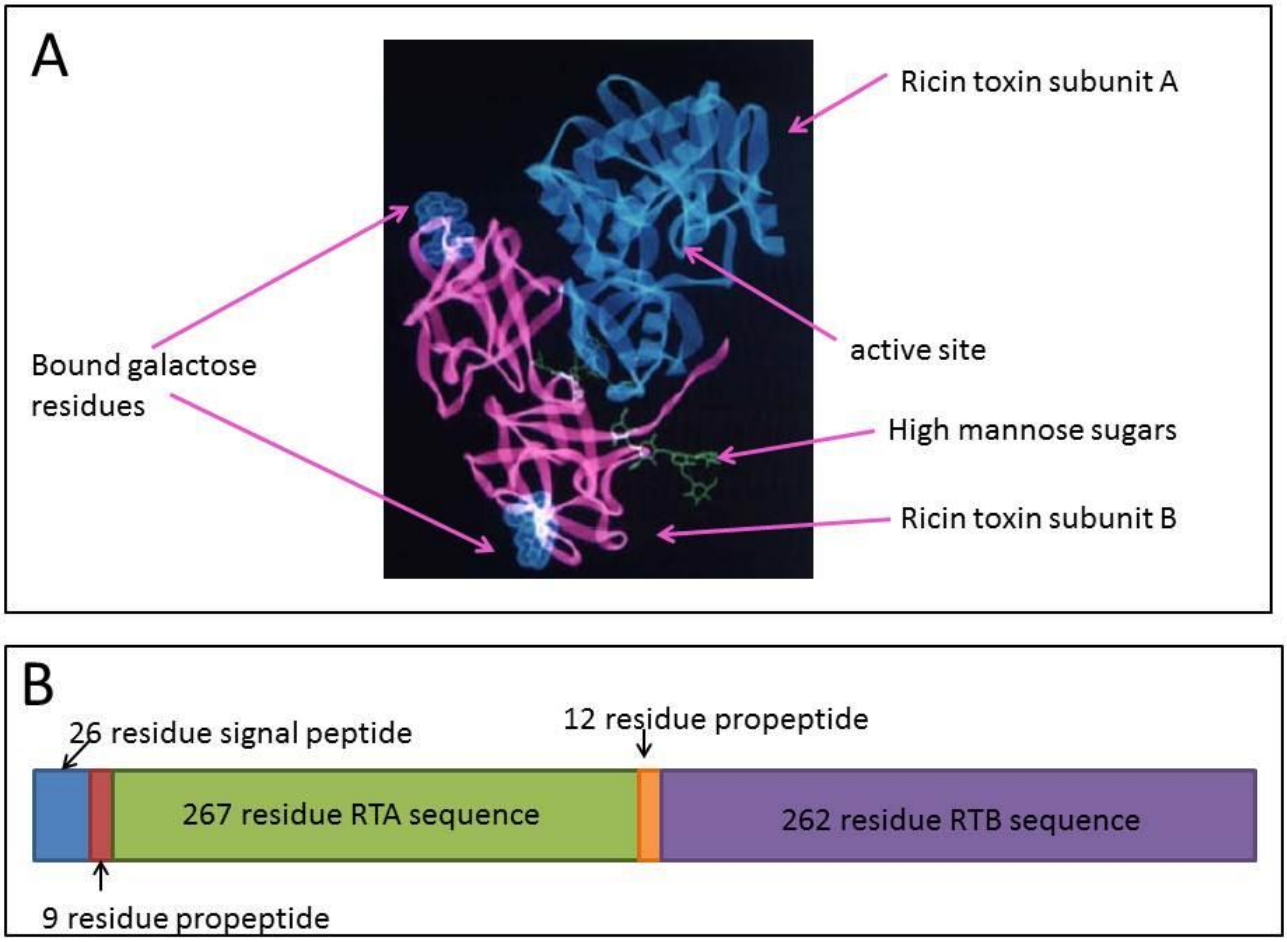



Figure 1.2.1: Structure of ricin and proricin.

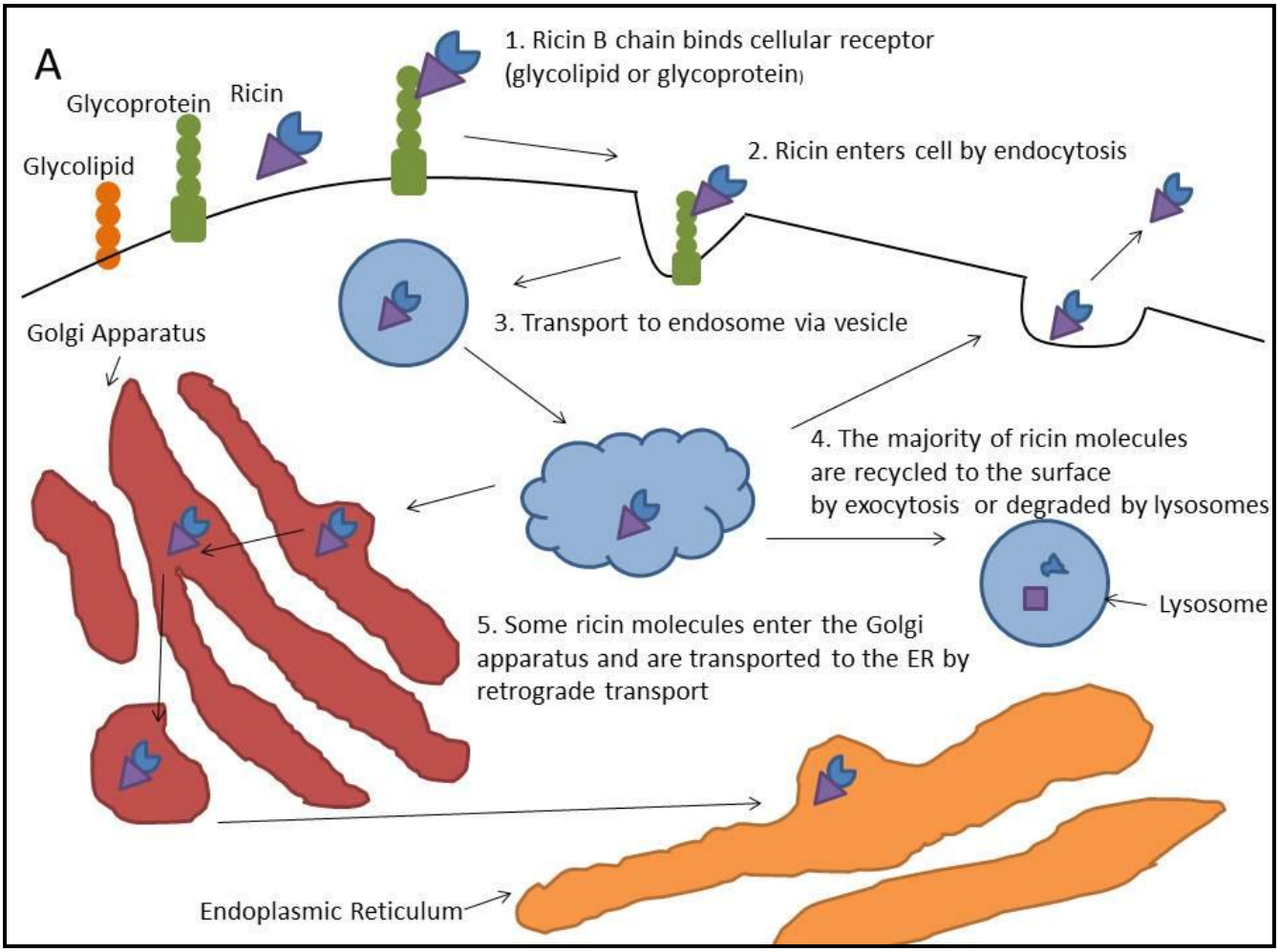
(A) Molecular structure of ricin. Reprinted with permission from John Wiley and Sons: Proteins: Structure, Function and Bioinformatics (Rutenber and Robertus 1991).
(B) Sequence of proricin peptide including the signal peptide and propeptides not found in mature ricin.

#### 1.2.3.2 Ricin toxin B: Getting into the cell

The B chain of ricin is responsible for binding to the cell surface molecules and moving the toxin all the way from the cell surface to the ER. It has been noted that the uptake of ricin is slow (approximately 10% per hour), which helps to explain the lag time between ricin exposure and toxic effects (Doan 2004; Olsnes 2004; Spivak and Hendrickson 2005). RTB is a lectin and binds galactosides with β 1-4 linkages which are present on a wide range of surface glycoproteins and glycolipids (Lord, Jolliffe et al. 2003; Lord, Roberts et al. 2005). As there are so many different molecules containing this terminal galactose, ricin binds not only to most cell types, but also binds all over the cell surface as the average cell has millions of potential binding sites (Sandvig, Grimmer et al. 2000; Doan 2004; Olsnes 2004). By binding many different types of receptors ricin can also take advantage of many different methods of endocytosis to gain access to the cell. Endocytosis of ricin was first visualized in clathrin coated pits, but since then other clathrin-independent methods have also been observed (Lord, Jolliffe et al. 2003; Olsnes 2004).

The transportation of ricin from the cell surface to the ER is summarized in Figure 1.2.2A. Following endocytosis ricin is transported to early endosomes, where the majority of the toxin is recycled back to the cell surface or transported to lysosomes for degradation. Only a small fraction of endocytosed ricin (approximately 5%) is transported to the *trans*-Golgi network and further to reach the cytoplasmic target (Sandvig, Grimmer et al. 2000; Lord, Jolliffe et al. 2003; Olsnes 2004). It remains unclear how ricin is transported from endosomes to the Golgi apparatus and this process could involve many different pathways, as the toxin is capable of

binding many different surface molecules.  Once reaching the trans-Golgi network ricin is transported retrogradely through the Golgi apparatus to the ER.


A
Glycoprotein
Ricin
Glycolipid
1. Ricin B chain binds cellular receptor
(glycolipid or glycoprotein)
2. Ricin enters cell by endocytosis
3. Transport to endosome via vesicle
Golgi Apparatus
4. The majority of ricin molecules
are recycled to the surface
by exocytosis or degraded by lysosomes
Lysosome
5. Some ricin molecules enter the Golgi
apparatus and are transported to the ER by
retrograde transport
Endoplasmic Reticulum

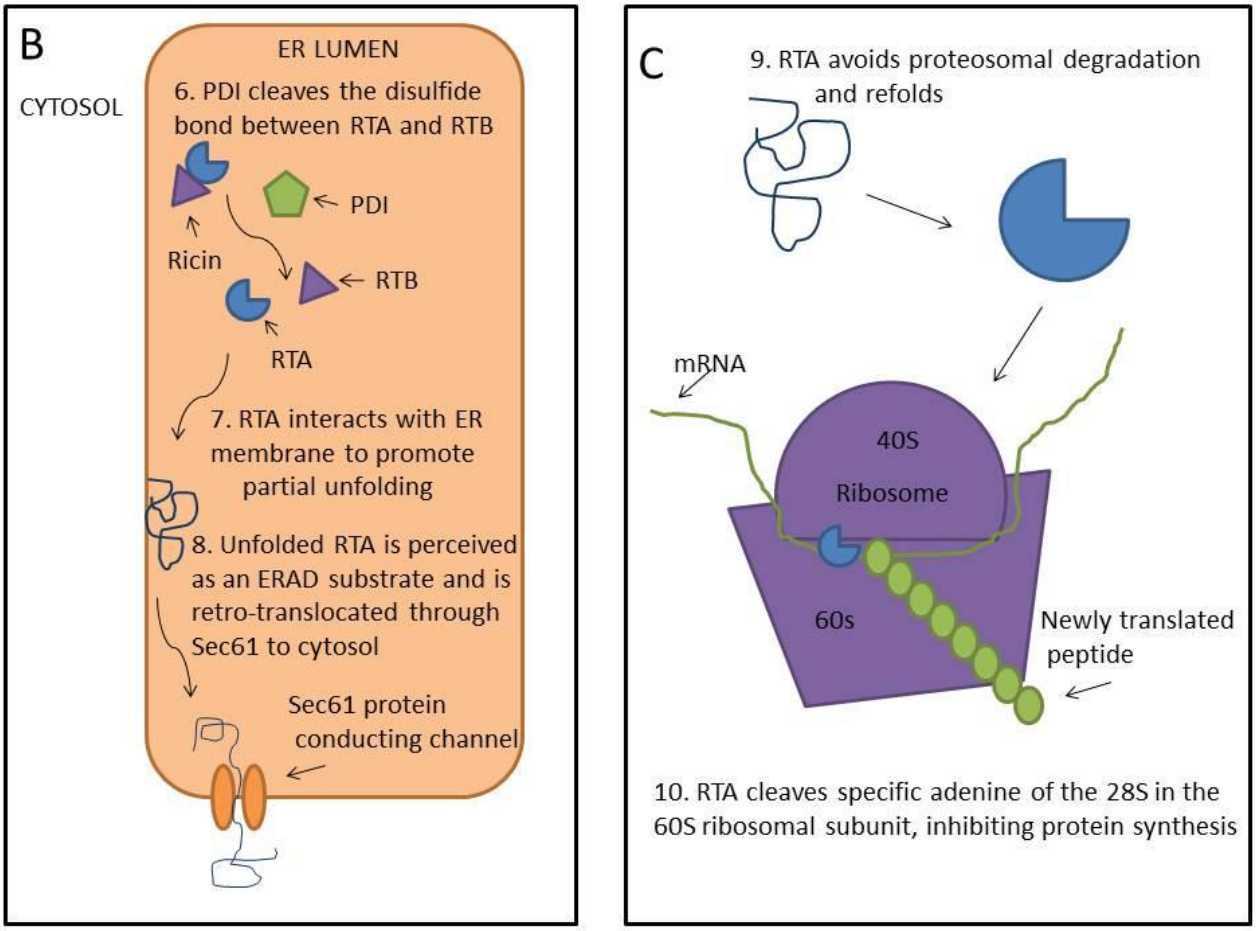



Figure 1.2.2: Summary of ricin activity

(A) Ricin movement from cell surface to ER (B) Retrograde transport of ricin through the ER (C) RTA glycosidase activity in the cytosol inhibits protein synthesis.

Adapted from Audi *et al.* 2005 and Lord, Roberts and Lencer 2006.

Ricin along with other closely related toxins such as cholera toxin, shiga toxin and *Pseudomonas* exotoxin A take advantage of pre-existing protein transportation methods to reach their cytoplasmic targets, with shiga toxin being the first demonstrated to use retrograde transportation to the ER (Sandvig, Grimmer et al. 2000). Shiga and cholera toxins contain C-terminal KDEL sequences which bind to protein sorting receptors and move proteins from the Golgi apparatus to the ER (Lord, Roberts et al. 2005). The mechanism used by ricin for this retrograde transportation is unclear as ricin does not contain a KDEL sequence. It is believed ricin may use calreticulin as a chaperone to reach the ER, as this protein contains a KDEL sequence as well as free galactose residues to which RTB could bind (Olsnes 2004; Lord, Roberts et al. 2005).

Once in the ER, ricin (as well as cholera toxin, shiga toxin and *Pseudomonas* exotoxin A) utilizes the host cell ER-associated protein degradation (ERAD) pathway to cross the ER membrane and reach its target (Figure 1.2.2B). The ERAD pathway is a stringent control system that recognizes misfolded and unassembled proteins in the ER and transports them to the cytosol for proteasome degradation (Sandvig and van Deurs 2002; Lord, Roberts et al. 2005). As seen in Figure 1.2.2B, inside the ER the disulfide bond is reduced separating the two ricin subunits after which, RTA is partially unfolded in order to be perceived as an ERAD substrate. It is believed that the disulfide bond is reduced by ER protein disulfide isomerase (PDI) and once separated from RTB, exposed surfaces of the A chain can then interact with the ER membrane to promote partial unfolding (Lord, Roberts et al. 2005). Once unfolded RTA is retrotranslocated to the cytosol by Sec61, an ERAD pathway protein conducting channel (Olsnes 2004; Bigalke and Rummel 2005; Lord, Roberts et al. 2005). After translocation most ERAD substrates are targeted for degradation by poly-ubiquitination of lysine residues, but ricin is able to avoid

degradation as it has unusually low lysine content. Studies have shown that the addition of lysyl residues to RTA not only increases its susceptibility to degradation but also reduces toxicity (Lord, Roberts et al. 2005). After this long journey from the cell surface through the Golgi apparatus and ER to the cytosol, the refolded biologically active ricin A chain is able to act on its target (Figure 1.2.3C).

#### 1.2.3.3 Ricin toxin subunit A: Ribosome inactivating protein

The mechanism of toxicity for ricin was first discovered by Lin et al. in 1970 in experimental tumour cells. The researchers set out to determine the mechanism of ricin cytotoxicity by investigating its effect on protein, DNA and RNA biosynthesis. These early studies observed that protein synthesis was inhibited followed by a decrease in DNA, while ricin had no effect on RNA levels. Studies were then conducted to examine the method of protein synthesis inhibition, ruling out an effect on glucose metabolism, cellular respiration and amino acid uptake, leading the researchers to believe ricin acted on protein synthesis machinery (Lin, Liu et al. 1971). Later studies determined ricin acts on ribosomes with the exact target being the 28S rRNA of the 60S subunit of eukaryotic ribosomes. The glycosidase activity of RTA was first described by Endo *et al*, as they demonstrated RTA cleavage of an N-glycosidic bond within an exposed loop of 28s rRNA (Endo, Mitsui et al. 1987; Endo and Tsurugi 1987). This residue was found to be a specific adenine (A4324 in rats) within a universally conserved sequence known as the sarcin-ricin loop which is associated with the binding of elongation factors (Lord, Jolliffe et al. 2003; Doan 2004; Olsnes 2004; Audi, Belson et al. 2005; Lord, Roberts et al. 2005). After cleavage, the phosphodiester bonds surrounding this depurinated adenine residue are then highly susceptible to hydrolysis which prevents the binding of elongation factors

necessary for protein chain elongation catalysis (Doan 2004). As the ribosomes are unable to bind elongation factors and therefore synthesize proteins, the cell eventually dies.

More recent research has further investigated the active site of RTA determining the molecular interactions between the glycosidase enzyme and ribosome. The active site of RTA is found in a pronounced cleft, with tyrosine residues 80 and 123 (Y80 and Y123) as well as glutamic acid residue 177 (E177) and arginine residue 180 (R180) being of particular importance to enzymatic activity (Lord, Jolliffe et al. 2003). As the rRNA substrate binds in the active site the target adenine becomes sandwiched between the two tyrosine rings (Y80 and Y123) of RTA (Musshoff and Madea 2009). While the target adenine interacts with Y80 and Y123, the ricin residue R180 is able to protonate the leaving group of the adenine N-3 which facilitates electron flow cleaving the bond between N-9 of the adenine and C-1' of the ribose (Lord, Jolliffe et al. 2003). The role of ricin E177 is to stabilize the oxycarbonium ion produced on the ribose as a result of bond cleavage (Lord, Jolliffe et al. 2003).

This single and specific depurination by ricin is able to completely and irreversibly inactivate the ribosome. Although only a small percentage of ricin molecules eventually reach the cytoplasmic target, only a single molecule of RTA is necessary to cause cell death. This is because the A chain is able to inactivate approximately 2000 ribosomes per minute, which is faster than the cell can produce new ribosomes (Olsnes 2004). Cell death due to protein synthesis inhibition is the major cause for symptoms associated with ricin poisoning. Many of the symptoms observed are caused by the substantial death of endothelial cells, which results in fluid leakage and tissue edema known as vascular leakage syndrome (Bradberry, Dickers et al. 2003). It is also thought that other mechanisms of toxicity may contribute to symptoms

associated with ricin poisoning. Ricin is believed to act on apoptosis pathways and cause cell membrane damage, alteration of membrane structure as well as trigger the release of cytokine inflammatory mediators (Audi, Belson et al. 2005). Studies conducted in both animals and cell culture have detected biochemical disturbances associated with oxidative stress indicating it may be a contributing factor to ricin induced cytotoxicity (Sehgal, Kumar et al. 2011).

## 1.3 Bioterrorism and Area Decontamination

### 1.3.1 Bioterrorism

Biological weapons can be defined as living organisms or material derived from them which are used for hostile purposes to cause harm or produce casualties in humans, animals or plants (Clarke 2005; Hicks, Hartell et al. 2005). Bioterrorism is the use of these biological weapons to create fear in a community as part of a religious, political or ideological goal. Biological weapons are often an appealing weapon for terrorist groups as they are relatively easy to acquire in comparison to other weapons, easy to disseminate, and have the ability to cause widespread fear and panic far beyond the actual physical damage caused (Zapor and Fishbain 2004). These weapons are also difficult to detect as they are invisible, silent and tasteless in comparison to nuclear, chemical and explosive weapons. They are also significantly less expensive to produce, costing approximately 0.05% that of other weapons able to cause similar casualties (Hawley and Eitzen 2001). Although the probability of such an event occurring is low, the consequences associated with a biological attack are severe, causing much media attention in recent years.

The USA Center for Disease Control and Prevention (CDC) has categorized biological agents (select agents) which have the potential for use in bioterrorism based on their severity. Category A includes the highest priority agents which pose the greatest risk and includes; anthrax (*Bacillus anthracis*), tularemia (*Francisella tularensis*), smallpox (variola major), botulinum neurotoxin (produced by *Clostridium botulinum*), bubonic plague (*Yersinia pestis*), and viral haemorrhagic fevers (including filoviruses such as Ebola and Marburg and arenaviruses such as Lassa). These organisms and toxins are categorized as such because they are easy to disseminate and transmit as well has being associated with high mortality rates and the ability to cause public panic and social disruption (CDC). Category B agents are defined as being moderately easy to disseminate and are associated with moderate rates of morbidity and low rates of mortality (CDC). Included in this category are; ricin toxin (*Ricinuns communis*), Brucellosis (*Brucella* species), Q fever (*Coxiella burnetii*), Staphylococcal enterotoxin B, as well as bacterial species considered as food and water safety threats. Category C agents are emerging pathogens which have the potential to be engineered for mass dissemination and are readily available with potential to cause a major health impact (CDC). These include emerging diseases such as SARS, Hantavirus, Nipah virus and certain strains of influenza. Similar Canadian definitions are also outlined in the 2009 Human Pathogens and Toxins Act (HPTA) (DepartmentofJustice 2009).

#### 1.3.1.1 History of Biological Weapons and Bioterrorism

Although the media attention given to biological weapons is relatively new, pathogenic organisms have been used to deliberately spread disease for centuries. Human civilizations have used fomites, cadavers, carcasses and other contaminated items in order to directly or indirectly

cause disease in their enemies (Clarke 2005). Corpses were used to contaminate drinking water in ancient Greek and Roman times; in the 14th century plague victim corpses were thrown over city walls by Tartar forces during the siege of Kaffa; and the British forces used smallpox infected blankets and clothing items to infect Native American populations during the 18th century (Clarke 2005). During World War II, many countries such as Japan, the former Soviet Union, USA, Canada and the UK developed biological weapons programs in order to research and develop ways of using these agents against their enemies. The former Soviet Union program is believed to have been the largest of its kind in the world, with eight storage and production facilities producing hundreds of tons of weapons-grade anthrax (Hicks, Hartell et al. 2005). More recent examples of biological weapons use include the Bhagawan Shree Rjneesh cult attempt in 1984. This is considered to be the largest attack in the USA and involved *Salmonella typhimurium* contamination of restaurant salad bars in Oregon in an attempt affect the local election by keeping voters home, resulting in 751 infected individuals (Clarke 2005). Over time world leaders have realized the unpredictable and often uncontrollable global outcomes associated with the use of biological weapons and have attempted to restrict their use. The first international attempt to restrict the use of biological weapons was the 1925 Geneva protocol. This was followed by the Biological Weapons Convention (BWC) which was ratified in 1975 and prohibits the acquisition of biological materials for hostile purposes (Clarke 2005). Member countries were required to destroy all stockpiles of biological weapons and limit biological research programs to defensive measures.

#### 1.3.1.2 Toxins as Biological Weapons

Several toxins are included in the CDC select agent list as they have great potential for use as a biological weapon. Toxins are defined as small molecules produced inside living organisms which are capable of causing disease or harm to humans or other organisms (Hicks, Hartell et al. 2005). Toxins can be difficult to classify as they fall somewhere between chemical and biological weapons. Toxins differ from chemical weapons as they are non-volatile, do not absorb through the skin and are much more lethal per unit weight (Hicks, Hartell et al. 2005). These molecules also cannot be categorized with biological agents as they do not have the ability to reproduce, they are not contagious and they are not always associated with a delay in the onset of symptoms as are microorganisms (Hicks, Hartell et al. 2005).

Toxins pose a great threat for use in a bioterrorism attack as they are easily attainable from the environment and can be used either directly as purified proteins or indirectly using the pathogen producing them as the delivery vehicle. As preformed molecules, toxins are fast acting and can be very potent even at low concentrations causing morbidity and mortality that is largely untreatable. Toxins can be easy to disseminate and the delivery method is often one of the most important aspects determining the potential of a toxin for use as a biological weapon (Clarke 2005). Contamination of food and water supplies would likely be the most effective method of delivery as these approaches have the ability to affect large numbers of individuals (Clarke 2005). Cruise missiles have been suggested as the ideal mode of delivery for causing inhalational illness as this method results in a cloud of aerosolized particles close to the ground (Clarke 2005).

#### 1.3.1.3 Anthrax and Bioterrorism

*B. anthracis* is perhaps the most notorious agent to be developed for use as a biological weapon. Anthrax had become the weapon of choice by the early 19th century and by the Cold War era it was the organism most commonly developed in state sponsored biological weapons programs (Clarke 2005). Anthrax has continued to maintain this position as the leading biological weapon due to its high mortality rate, the global availability of the bacteria from the environment as well as the stability of the *B. anthracis* spore. As person to person transmission of anthrax disease is rare, spread beyond the target population is unlikely, which is ideal for use as a biological weapon. The most effective method of delivery of anthrax is the dormant spore. Although the isolated anthrax toxins could be used to cause illness, the spore provides the best means of spreading the disease due to its stability.

One of the earliest occasions of anthrax spore biological weapon development was the Japanese research program Unit 731 from 1932-1945. During this time, hundreds of thousands of prisoners were infected with anthrax spores killing at least 10,000 individuals as well the stockpiling of 900lbs of anthrax with the intention of using it in fragmentation munitions (Hicks, Hartell et al. 2005). Anthrax was also developed by the USA and UK as a biological weapon known as Agent N during World War II. Gruinard Island in Scotland was used by the British army to test anthrax for use as a biological weapon, including the N-bomb, a bomb filled with anthrax spores. The N-bomb was never used and Gruinard Island was later decontaminated with formaldehyde. The event most responsible for the current fame of anthrax as a biological weapon was the release of anthrax spores in the US mail system in 2001. This single event

resulted in a massive increase in the American biodefense budget, rising from $137 million in 1997 to $6 billion per year by 2001 (Franz 2009).

#### 1.3.1.4 Ricin and Bioterrorism

The lethality of ricin has been long recognized, with reports of its use for homicidal purposes going back to ancient times (Olsnes 2004). This toxin has been a weapon of choice for bioterrorist groups and extremist individuals due to its extreme potency, relative availability and ease of production (Lord, Jolliffe et al. 2003; Zapor and Fishbain 2004; Bigalke and Rummel 2005; Hicks, Hartell et al. 2005; Spivak and Hendrickson 2005). The isolation of crude ricin is relatively simple requiring only knowledge of basic chemistry techniques, with protocols for extraction available on the internet. Contaminations of food and water supplies as well as aerosol release of the toxin are the most likely means of delivery. Although inhalation of ricin is 3 times more toxic than exposure by oral route, purified particles as well as an aerosolization dispersal device would be required (Bradberry, Dickers et al. 2003; Audi, Belson et al. 2005).

Ricin has also been considered for use in warfare by several countries including the UK and USA who were reported to have studied its potential during World War I and developed weapons grade ricin, code named Compound W during World War II (Olsnes 2004; Audi, Belson et al. 2005; Hicks, Hartell et al. 2005; Spivak and Hendrickson 2005). The W-bomb was tested during the 1940s as a potential means of causing inhalational ricin poisoning, but was never used in combat. After the ratification of the BWC these research programs were banned although research was continued in the former Soviet Union and Iraq, the latter rumoured to have produced weapons grade ricin and tested the product on animals and in artillery shells until the late 1980s (Audi, Belson et al. 2005; Bigalke and Rummel 2005).

Undoubtedly, one of the most famous uses of ricin was the 1978 assassination of Bulgarian exile Georgi Markov in London. The murder was believed to be supported by the Soviet KGB, and utilized a spring loaded needle filled with a small pellet of ricin fired from an umbrella into the thigh of Markov (Spivak and Hendrickson 2005; Musshoff and Madea 2009). Markov became ill within a few hours exhibiting non-specific symptoms and was later admitted to hospital where his condition rapidly declined, concluding with his death four days after the injury. Ricin was believed to be the cause of death due to the symptoms and high level of toxicity for the small dose utilized.

### 1.3.2 Area Decontamination Methods

In the event of an intentional or accidental release of a biological weapon the surrounding area can become contaminated. To protect the public from further exposure, buildings, equipment and first responder vehicles must be decontaminated. The 2001 intentional release of anthrax spores in the US postal system has highlighted the need for technologies capable of effectively decontaminating large spaces to assist in the clean up after these bioterrorism related attacks. After these events, five mail facilities and two office buildings required decontamination; this was accomplished using hydrogen peroxide, chlorine dioxide and formaldehyde (Rogers, Richter et al. 2010). Since this time there has been an increase in research and validation of area decontamination methods such as vapourous hydrogen peroxide (VHP) and gaseous chlorine dioxide (GCD), particularly in their ability to inactivate *B. anthracis* spores.

Surface decontamination using a liquid disinfect is often the simplest method and is ideal for decontaminating small areas. When large areas, complex equipment and a variety of

different surfaces require decontaminating gaseous methods or fumigants are often a better choice. In these situations liquid disinfectant wipe down techniques can be time consuming and difficult to standardize (Krause, McDonnell et al. 2001). Gas decontamination methods are advantageous in their ability to cover large surface areas and more difficult to reach areas (Rogers, Choi et al. 2008).

There are many factors that can influence the efficacy of the different gaseous decontamination methods. The microorganisms or biological material present and their concentration and level of soiling are important as different organisms have different levels of resistance to inactivation, with bacterial spores being the most resistant. The environmental conditions of the area must be monitored as the temperature and relative humidity can influence efficacy depending on the fumigant used. The most important factor influencing efficacy is the concentration of the fumigant and length of the exposure. The general trend is the higher the fumigant concentration the shorter the exposure length required, and the lower the fumigant concentration the longer the exposure required to inactivate microorganisms present.

#### 1.3.2.1 Vaporous Hydrogen Peroxide

Vapor based hydrogen peroxide decontamination was first developed in the 1980s, commercialized in the early 1990s (Rickloff and Graham 1989; Graham and Rickloff 1992) and originally designed for use in pharmaceutical clean rooms (Davies, Pottage et al. 2010). Since this time the technology has been further applied to the decontamination of animal rooms, biological safety cabinets (BSCs), containment laboratories, and pass-through boxes (Heckert, Best et al. 1997; Krause, McDonnell et al. 2001; Kahnert, Seiler et al. 2005; Krishnan, Berry et al. 2006; Hall, Otter et al. 2007; Fey, Klassen et al. 2010) and is considered to have great

potential for use in decontaminating buildings affected by bioterrorism. Hydrogen peroxide is an oxidizing agent that works by producing highly reactive hydroxyl radicals which attack cell membranes, DNA and proteins causing cell death. A recent study (Finnegan, Linley et al. 2010) investigated the mode of action of several disinfectants including vaporous hydrogen peroxide, and found the vapor form to be much more effective at denaturing proteins than the liquid form.

The STERIS vaporous hydrogen peroxide (VHP) system generates a vapor by flash vaporizing a 35% aqueous solution of $H_2O_2$. With this system it is important to reduce the relative humidity (RH) of the enclosure to <40% to prevent condensation of the vapor, avoiding corrosion and increasing vapor distribution. The VHP decontamination cycle begins with the dehumidification phase where the relative humidity of the area is lowered to <40%. The conditioning phase follows where high volumes of hydrogen peroxide are injected into the area over a short period of time. After this the vapor is injected at a set rate to achieve a steady concentration of hydrogen peroxide for the length of the decontamination phase. After decontamination the area is aerated by cycling the air through the generator to be decomposed to water and oxygen or evacuated using the area's exhaust system.

Several studies have been conducted on the efficacy of VHP and the application of this vapor based decontamination system in containment laboratory settings. VHP has been successfully used to decontaminate CL3 laboratories (Kahnert, Seiler et al. 2005; Krishnan, Berry et al. 2006; Hall, Otter et al. 2007) and was effective in eliminating *Geobacillus stearothermophilus* spores and *Mycobacterium tuberculosis*. One study has also been conducted to validate the use of VHP for decontamination of a CL4 laboratory (Stansfield, Fey et al. 2008). Research has also proven VHP to be effective in decontaminating animal rooms and the animal

caging systems contained within (Krause, McDonnell et al. 2001). VHP has also been investigated for use in decontaminating BSCs. One more recent study (Fey, Klassen et al. 2010) evaluated VHP decontamination of a BSC in a worst case scenario. After several unsuccessful cycles, a repeatable cycle and set of conditions were developed indicating VHP could successfully be used to decontaminate BSCs. The unsuccessful trials highlight the need to thoroughly validate decontamination protocols used for complex equipment such as BSCs (Fey, Klassen et al. 2010). VHP was also used to decontaminate the Hart Senate building after the 2001 anthrax attacks (Richter, Wendling et al. 2009).

VHP has been proven effective against a variety of organisms including bacterial spores such as *B. anthracis*, vegetative bacteria such as *Y. pestis* and *F. tularensis*, mycobacteria including *M. tuberculosis*, viruses and prions (Heckert, Best et al. 1997; Johnston, Lawson et al. 2005; Kahnert, Seiler et al. 2005; Meszaros, Antloga et al. 2005; Rogers, Sabourin et al. 2005; Fichet, Antloga et al. 2007; Hall, Otter et al. 2007; Rogers and Choi 2008; Rogers, Richter et al. 2008; Rastogi, Wallace et al. 2009). Like with most disinfectants, bacterial spores have proven to be the most resistant to decontamination. To date, *G. stearothermophilus* has been identified as the most resistant to hydrogen peroxide and as such is used as a biological indicator for VHP decontaminations (Klapes and Vesley 1990; Meszaros, Antloga et al. 2005). Research has indicated that organisms which produce superoxidase dismutase, catalase and other peroxidases have increased resistance to VHP thus requiring longer exposure times, as these enzymes can break down the $H_2O_2$ (Kahnert, Seiler et al. 2005). No published data is available on the efficacy of VHP against biological toxins.

The factor considered to have the greatest influence on the efficacy of VHP is the level of soil load or organic material present. Organic soil load, such as dried blood had been found to decrease the efficacy of VHP against viruses, vegetative bacteria and bacterial spores (Heckert, Best et al. 1997; Meszaros, Antloga et al. 2005; Krishnan, Laframboise et al. 2006; Pottage, Richardson et al. 2010). This decreased efficacy can be partly due to the high concentrations of catalase present in some organic soil loads (especially in blood), but is also due to fact that hydrogen peroxide will react with any oxidisable material present and this can decrease the amount of hydrogen peroxide available to kill microorganisms (Meszaros, Antloga et al. 2005; Davies, Pottage et al. 2010). These findings have highlighted the importance of precleaning when using VHP for decontaminating. The cleaner the surfaces in an area the more reproducible the decontamination (Meszaros, Antloga et al. 2005).

Another factor that plays a role in decontamination efficacy is the surface material present. Several studies have been conducted investigating the efficacy of VHP on a variety of materials (Rogers, Sabourin et al. 2005; Krishnan, Laframboise et al. 2006; Rogers and Choi 2008; Rogers, Richter et al. 2008; Rastogi, Wallace et al. 2009). These studies are concentrated on building materials in order to ascertain the effectiveness of VHP in decontaminating buildings affected by bioterrorism. All studies found decontamination to be more effective on non-porous materials than on porous materials, with concrete and cinder block being the most difficult to decontaminate. Organisms on a non-porous material are easily reached by VHP on the surface whereas the vapor must penetrate porous materials in order to reach organisms which have become embedded in cavities located within the material (Rogers, Sabourin et al. 2005). The hydrogen peroxide may also interact with the material, decomposing the chemical before it is able to reach the microorganisms. This is the case for the ineffectiveness of VHP on concrete

surfaces where the iron present in concrete is believed to decompose the hydrogen peroxide (Krishnan, Laframboise et al. 2006). Porous materials have also been found to require extended aeration periods after decontamination due to off gassing (Davies, Pottage et al. 2010). With the most data available among fumigants on material compatibility, VHP has been proven to be compatible with wide variety of materials although studies have indicated it is incompatible with nylon, neoprenes, some anodized aluminum and some epoxides (Rogers, Choi et al. 2008; Davies, Pottage et al. 2010). Decontaminations in laboratories containing sensitive equipment such as computers, cameras, incubators and other heat sensitive equipment have indicated VHP to be compatible with such equipment and no damaging effects were observed (Heckert, Best et al. 1997; Krause, McDonnell et al. 2001; Hall, Otter et al. 2007; Davies, Pottage et al. 2010).

One of the major advantages of hydrogen peroxide is its safety in comparison to other fumigants such as formaldehyde and ozone. Hydrogen peroxide is considered to be less toxic as it readily breaks down into water and oxygen, with no toxic by-products or residues (Rogers, Choi et al. 2008; Davies, Pottage et al. 2010). The VHP decontamination technology also allows automated cycles and the ability to control and monitor conditions of the decontamination area, thus increasing safety for the users. As well, hydrogen peroxide is used at lower concentrations (750-1500 ppm) and shorter contact times (1-4 hours) than formaldehyde, which is normally used at 8000-10000 ppm for twelve hours (Czarneski 2007).

#### 1.3.2.2 Gaseous Chlorine Dioxide

Chlorine dioxide in aqueous form has long been used for its disinfecting properties, but the gaseous form was first registered as a chemosterilizing agent in 1984 and registered with the EPA in 1988 (Czarneski and Lorcheim 2005). One of the earliest studies on gaseous chlorine

dioxide (GCD) investigated the conditions necessary for sporicidal activity (Jeng and Woodworth 1990). Since this time GCD has been further investigated for its use in decontaminating high containment laboratories, BSCs, pharmaceutical research facilities and animal facilities as well as buildings affected by bioterrorism. Like hydrogen peroxide, chlorine dioxide functions as an oxidizing agent. Chlorine dioxide is a very selective oxidant with two and a half times the oxidizing power of chlorine (Davies, Pottage et al. 2010). Due to the selective oxidizing nature of chlorine dioxide, it only reacts with molecules that are highly reduced (alcohols, aldehydes, ketones, tertiary amines and sulfur containing amino acids) which causes its activity to be much less influenced by organic soil loads (Luftman, Regits et al. 2008)

With a boiling point of 11°C, chlorine dioxide is a gas at room temperature and is therefore considered a true gas (Czarneski 2007).

GCD is a synthetic gas that is stable for only a short period of time and is generated at time of use by a few different methods (Luftman, Regits et al. 2008). In the dry method, GCD is generated by passing chlorine gas through cartridges of sodium chlorite producing pure gas. This dry method is utilized in the generators produced commercially by ClorDiSys Solutions. Decontamination cycles consist of five phases beginning with pre-conditioning, in which the relative humidity of the area is raised to >70%. After this is the conditioning phase where the relative humidity is monitored to ensure it is maintained. GCD is generated and injected into the area during the charging phase, after which the GCD concentration is maintained for the length of the exposure phase. Most generators contain sensors which monitor the GCD concentration in real time so if the concentration drops due to absorbance by celluoistic materials or leakage, more gas can be injected to maintain the set concentration (Czarneski and Lorcheim 2005). After the exposure time, all gas generation is stopped and the space is aerated to remove any

remaining gas. Often the air is passed through a scrubber to decompose all remaining chlorine dioxide.

Extensive research has been conducted investigating the use of GCD in the agriculture and food processing industries, with its use in high containment facilities and for buildings affected by bioterrorism only beginning in recent years resulting in a limited number of published studies available. GCD has successfully been used to decontaminate large areas such as an 18,000 $ft^2$ animal research facility and a 17,000 $ft^2$ animal hospital (Luftman, Regits et al. 2006; Czarneski 2009). In both cases, sealing the facility proved to be the most difficult aspect of the decontamination. GCD has also been validated for decontaminating BSCs using a protocol developed in conjunction with NSF International (Luftman, Regits et al. 2008). After the 2001 anthrax release in the US mail system, GCD was used to decontaminate several mail processing buildings as well as office buildings (Rastogi, Ryan et al. 2010).

Due to its strong oxidizing abilities, GCD is effective against a wide variety of organisms. Many studies have found GCD effective against food-borne pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* in applications in the food processing industry (Mahmoud, Bhagat et al. 2007; Mahmoud, Vaidya et al. 2008; Bhagat, Mahmoud et al. 2010; Bhagat, Mahmoud et al. 2010; Trinetta, Morgan et al. 2010). GCD is also effective against viruses even at relatively low concentrations with extended exposures (Morino, Fukuda et al. 2009; Sanekata, Fukuda et al. 2010). Most efficacy studies on GCD for use in high containment laboratories and bioterrorism related events are conducted against *Bacillus* spores as they are the most resistant to decontamination. One study (Luftman and Regits 2008) was undertaken to determine which spore species was the most resistant and best for use as a

biological indictor. The study focused on *Bacillus atrophaeus* and *G. stearothermophilus* based on previous studies (Jeng and Woodworth 1990) and because these are the organisms most resistant to formaldehyde and hydrogen peroxide, respectively. The authors found the two organisms to have similar resistance, dependent on conditions, but *G. stearothermophilus* decontaminations resulted in more variability making *B. atrophaeus* a better choice for a biological indicator (Luftman and Regits 2008). There are currently no published studies investigating the efficacy of GCD against biological toxins.

The condition found to influence GCD efficacy the most is the relative humidity (RH) of the decontamination enclosure. This was first noted by Jeng and Woodworth (1990) when they observed the sporicidal activity of GCD was enhanced by prehumidification to >70%. Research has shown that RH levels >80% increase efficacy resulting in much shorter exposure times, however, successful decontamination with lower humidity levels (50%) can be achieved with extended exposure times (Gordon, Krishnan et al. 2007). This might be ideal in situations where it is not possible to maintain high humidity levels and extended exposure times are not an issue. Relative humidity was found to be most important in cases where the challenge organism is in a dry state (Morino, Fukuda et al. 2009). The effects of challenge organism concentration and soil load on the efficacy of GCD have also been investigated. GCD was found to be less effective to spore concentrations greater than $1 \times 10^8$, but soil loads of serum up to 5% were not found to influence efficacy (Rastogi, Wallace et al. 2009). Others have also found soil load to not influence GCD efficacy as it does with hydrogen peroxide (Krishnan, Laframboise et al. 2006).

As with VHP, studies have been conducted investigating the efficacy of GCD on different surface materials commonly found in buildings. GCD was also found to have

decreased efficacy on porous materials such as wood, cinder block and paper (Han, Applegate et al. 2003; Krishnan, Laframboise et al. 2006; Rastogi, Wallace et al. 2009; Rastogi, Ryan et al. 2010). Greater variability in organism inactivation was also observed on porous materials; this being attributed to the microscopic pores and structural nonuniformity of complex materials (Rastogi, Ryan et al. 2010). Organisms on non-porous materials such as glass and stainless steel were found to require much shorter inactivation times (Han, Applegate et al. 2003; Krishnan, Laframboise et al. 2006; Rastogi, Wallace et al. 2009; Rastogi, Ryan et al. 2010). Material compatibility studies have found GCD to be compatible with most materials such as paper, wood, epoxy, plastics and stainless steel, but do indicate bleaching of some materials such as carpet (Rogers, Choi et al. 2008). Corrosion of surfaces has not been observed with the pure gas produced by gas generators and it is compatible with sensitive equipment and electronics.

Gaseous chlorine dioxide has excellent potential for decontaminating in high containment laboratories due to its rapid inactivation of a wide variety of pathogens even in the presence of organic soil loads. GCD is also considered to be a safer alternative to formaldehyde fumigation as it is non-carcinogenic and does not produce toxic by-products or residues. Gas generators also provide the safety of external control and monitoring of gas concentration. As well, GCD decontaminations require lower concentrations (360-1800 ppm) and shorter exposures (0.5-2 hours) than formaldehyde (Czarneski 2007).

## 1.4 Research Goals

Biological toxins including ricin and the lethal factor and protective antigen of *B. anthracis* are easily found in nature. Small quantities of these proteins can be lethal to humans, allowing these biological toxins to have great potential for use as bioweapons. In the event of an intentional or accidental release of a biological toxin, the surrounding area can become contaminated. To protect the public from further exposure, buildings, equipment and first responder vehicles must be decontaminated. Gaseous methods of decontamination such as VHP and GCD have been investigated for their use in decontaminating buildings affected by biological weapons. Studies have shown these methods to be effective against microorganisms, but very little work has been conducted to test their efficacy against biological toxins. Inactivation of biological toxins can be more difficult to test than microorganisms as they often require complex detection methods such as animal bioassays or the development of sensitive cellular assays. The goals of this research are to further develop, evaluate and modify previously described detection methods for testing the detoxification of ricin and the anthrax lethal toxin (LF and PA together), and to determine the efficacy of gaseous decontamination methods to inactivate these toxins. Findings from these studies will assist microbiologists and biosafety professionals in making decisions to manage these dangerous substances in order to protect the public and first responders as well as controlling the risk of infectious diseases.

# CHAPTER 2.0: MATERIALS AND METHODS

## 2.1 Toxins

Edema factor was not used for efficacy testing as it is not toxic to any cell lines and as such no *in vitro* cytotoxicity assays are available for detecting the toxin. Recombinant forms of anthrax lethal factor and protective antigen were purchased from List Biological Laboratories Inc. (Campbell, CA., USA). The proteins are produced separately by NIH licensed plasmids in avirulent *B. anthracis* lacking wildtype plasmids pX01 and pX02 and the ability to sporulate (ListBiologicalLaboratories 2011). Quality documented (QD) forms of the toxins were used, which undergo quality control testing for purity and activity for each lot produced. The purified and lyophilized toxins were resuspended in sterile purified water at a concentration of 1mg/ml and stored at -80°C until use. Toxins were aliquoted with enough for one experiment to prevent degradation from repeated freeze thaw cycles.

Purified ricin toxin extracted from Carmencita red cultivar of *R. communis* was acquired from the Modeling and Analysis Group at Defense Research and Development Canada (DRDC) Suffield. The protein was purified by previously described methods (Lin and Liu 1986). Mouse bioassay conducted by DRDC Suffield indicated an $LD_{50}$ of 10 µg/kg for the extracted and purified ricin. Upon receipt, the toxin was aliquoted at a concentration of 1mg/ml and stored at-80°C until use. All experiments with ricin were conducted in a CL3 laboratory.

## 2.2 Decontamination Efficacy Testing: Quantitative Carrier Test

A modified version of the Quantitative Carrier Test tier 2 (QCT-2) was used to conduct decontamination efficacy experiments. The QCT method, an ASTM international standard (E-

2111 and E-2197), was developed at the Centre for Research on Environmental Microbiology and is explained in detail in Springthorpe and Sattar (2003). This method includes two tiers, QCT-1 mostly used as a screening tool and the more stringent QCT-2, to test the efficacy of liquid disinfectants on surfaces contaminated with microorganisms. The QCT-2 methodology provides a realistic and stringent testing environment by utilizing a brushed stainless steel carrier and a dried inoculum containing the challenge organism and a soil load of BSA, tryptone and mucin (Springthorpe and Sattar 2005). The carrier surface contains scratches and irregularities commonly found on worn surfaces, which may prevent disinfect coming in contact with the test organism. The soil load employed in this procedure is approximately equivalent to 5-10% serum and mimics that of organic material commonly present with microorganisms which may neutralize disinfects and provide protection to microorganisms.

The basic QCT-2 procedure involves drying the test organism and soil load on the stainless steel carrier, followed by exposure to the test disinfectant for a specific time. The exposure time is then stopped by the addition of a neutralizing solution and any remaining organisms are enumerated. An unexposed positive control is used to establish the starting concentration of the test organism. The QCT-2 protocol can be adapted for use with all types of microorganisms including; bacteria, bacterial spores, viruses, fungi and mycobacteria (Springthorpe and Satter 2003).

This standard QCT-2 protocol was modified for use with gaseous decontamination. The modified procedure is shown in figure 2.2.1 and includes placing the carrier inoculated with dried toxin and soil load in the lid of an inverted Teflon vial (instead in the vial itself). During

exposure to the gas, the inverted vial is removed and the exposure is ended by placing the carrier into a vial containing the neutralizing solution.

## 2.3: Gaseous Decontamination

For each gaseous decontamination method and toxin combination, three separate tests were conducted. Each test included three unexposed controls and two replicates of each time point. As per QCT-2 protocol (Springthorpe and Satter 2003), carriers were inoculated with 10 µl of inoculum (170 µl of toxin added to 12.5 µl BSA, 17.5 µl tryptone and 50 µl mucin) and allowed to dry, resulting in approximately 6.67 µg of toxin per carrier. All exposures were conducted in the primary chamber of a class III biological safety cabinet (BSC) in a CL3 laboratory. The primary chamber (Figure 2.3.1a) was isolated by closing the exhaust and supply valves to contain the gas.

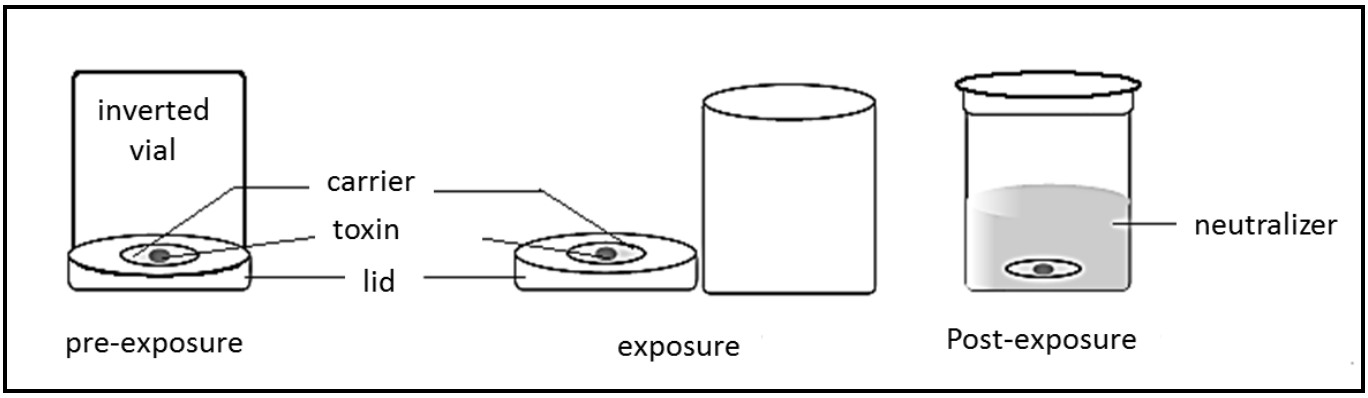



Figure 2.2.1: Modified QCT-2 protocol for exposure to gaseous decontamination.

Exposure to GCD was conducted using a Minidox-M generator (Figure 2.3.1b) from ClorDiSys (Lebanon, NJ., USA) connected to the class III BSC. The GCD decontamination cycle consisted of humidification to approximately 80% RH, conditioning, decontamination (5 mg/L GCD) and aeration phase. During the decontamination phase, toxins were exposed by removing the vial as per Figure 2.2.1. The activity of GCD was terminated using 1 ml of a neutralizing solution of 1% sodium pyrvuate in Opti-MEM media (Invitrogen, Burlington, Ont., Canada). Exposure to VHP was conducted using a VHP 1000ED generator (Figure 2.3.1c) from STERIS Corporation (Mentor, OH., USA) which was connected to the class III BSC. The decontamination consists of dehumidification, conditioning, decontamination and aeration phases. During the decontamination phase, once the concentration of VHP inside the enclosure reached a plateau (approximately 800-1000 ppm) exposure of the organisms began, as described above. The activity of the VHP was terminated using 1 ml of a neutralizing solution of 1% sodium pyruvate in Opti-MEM media. Following decontamination the exposed toxins were removed from the class III BSC and resuspended in the 1ml neutralizing solution by pipetting up and down and gentle scraping.

Exposure time points of one, five and ten minutes were used for anthrax toxins, LF and PA, as well as ricin. Based on preliminary testing a 30 minute time point was also added for ricin. Final time points of two hours for GCD and four hours for VHP were used (Czarneski 2007).


Figure 2.3.1: Equipment used in gaseous decontamination experiments.

(A) Primary chamber of class III BSC used for gaseous decontamination exposures, (B) front and back of ClorDiSys Minidox-M GCD generator, (C) front and back of STERIS VHP 1000ED generator.

## 2.4 Detection of Toxin Activity

As toxins are not living replicating organisms such as those for which the QCT methodology was originally designed, different methods were needed to determine detoxification after GCD and VHP exposure. The assay must detect not only the presence of the toxin but also its activity. For this reason a cytotoxicity assay was used which indirectly detects the activity of the toxins by measuring the cell death caused by the toxins. For both the anthrax toxins and ricin a colourimetric cytotoxicity assay was used, which detects the metabolism of MTT (2,5-diphenyl-tetrazolium bromide) by living cells. In viable cells the mitochondrial succinate dehydrogenase cleaves the yellow tetrazolium salt (MTT) forming purple formazan which can be detected spectrophotometrically. In non-viable cells the dehydrogenase is no longer functioning and no colour change occurs.

For the anthrax detection assay, both LF and PA toxins must be present in their active form in order to induce cytotoxicity. Mouse macrophage J774A.1 tissue culture cells were used for the anthrax toxin detection assay as they are one of the few cell lines for which the anthrax lethal toxin (LF and PA together) is cytotoxic. Cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Sigma-Aldrich, Oakville, Ont., Canada) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1mM sodium pyruvate (Invitrogen), 4mM L-glutamine (Sigma-Aldrich), 10mM HEPES buffer (Invitrogen) and 50 units penicillin-streptomycin (Invitrogen) (complete DMEM). Macrophage cells were grown from low passage liquid nitrogen stocks and passaged at least 5 times before using for cytotoxicity assays. The J774A.1 cell line was used for the cytotoxicity assay for up to 25 passages, after this time a fresh stock of cell was started from liquid nitrogen storage.

The day prior to conducting the assay, 96 well tissue culture plates of J774A.1 cells were prepared DMEM complete. Cells were detached from culture flasks (using a cell scraper) and suspended in DMEM complete media. Tissue culture plates were seeded with 1 x $10^5$ cells per well and incubated overnight at 37°C with 5% $CO_2$. The next day, unexposed stock LF and PA was diluted to 6.67 µg/ml and lethal toxin solutions were made for each test/time point by adding 250 µl of the exposed toxin to 250 µl of opposite stock toxin (ie. for tests of exposed LF toxin an equal amount of the stock (unexposed) PA toxin was added). Positive controls (dried unexposed toxins) were treated in the same manner. The concentration of both toxins in the lethal toxin solution positive control was 3.335 µg/ml. This lethal toxin solution was then further diluted in Opti-MEM as indicated in table 2.4.1. The medium was removed from the J774A.1 cell plates and the cells were washed with PSB. After washing, 75 µl of each lethal toxin solution (LF test time points and positive control and PA test time points and positive controls) was added to the cells in triplicate. Negative controls of media only were also added in triplicate. Cell plates were incubated for 4 hours at 37°C with 5% $CO_2$.

For detecting cell viability, 25 µl of MTT, 2,5-diphenyl-tetrazolium bromide (Sigma-Aldrich), at a concentration of 5mg/ml was added to each well of the cell plate. Plates were then incubated for 2 hours at 37°C and 5% $CO_2$. During this time viable cells will metabolize the MTT creating a visible colour change from yellow to purple. Non-viable cells will not metabolize the MTT and remain a yellow colour. After incubation, 100 µl of solubilization buffer prepared as previously described (Hering, Thompson et al. 2004) was added to each well to lyse the cells and dissolve the coloured precipitate. Plate were incubate overnight at 37°C with 5% $CO_2$ and the absorbance values were read at 570nm using a Multiskan Ascent plate reader (Thermo Scientific). Low absorbance values are indicative of the yellow MTT meaning

non-viable cells and higher absorbance values are indicative of the purple formazan meaning viable cells. Absorbance values of cells treated with media only (negative control) were taken as the maximum assay response (100% cell viability) and used to calculate the percent viability of cells treated with each dilution of anthrax toxin. Percent viability of the cells treated with undiluted exposed toxin was analyzed for statistically significant differences from the unexposed control using Microsoft Excel 2010. A two tail student's t-test assuming equal variance was used to compare each time point to the unexposed control.

The MTT cytotoxicity assay for ricin was conducted similarly with a few changes. Vero 76 (African Green Monkey Kidney) tissue culture cells were utilized for the assay and were cultured using DMEM with high glucose supplemented with 10% FBS and 100mM penicillin-streptomycin (DMEM complete). Vero 76 cells used for the cytotoxicity assay were passaged continuously and cell passages of 30-60 were used in this study. The day prior to the assay, 96 well tissue culture plates were prepared with $3.4 \times 10^4$ cells per well and incubated overnight at 37°C with 5% $CO_2$. Exposed ricin and controls were diluted similarly to the anthrax toxins in Opti-MEM, as indicated in Table 2.4.1. Culture media was removed from the Vero 76 cells and the cells were washed with PBS prior to adding 75 µl of each toxin dilution in triplicate. Cell plates were incubated for 22 hours at 37°C with 5% $CO_2$. The remainder of the assay was carried out in the same manner as with the anthrax toxins.

Table 2.4.1: Dilutions of lethal toxin solution and ricin as used in the MTT cytotoxicity assay

| Dilution | Approximate concentration of LF/PA in positive control lethal toxin solution | Approximate concentration in positive control of ricin |
|---|---|---|
| **Undiluted** | 3335 ng/ml | 6670 ng/ml |
| **0.5** | 1667 ng/ml | 3335 ng/ml |
| **0.2** | 667 ng/ml | 1334 ng/ml |
| **0.1** | 333.5 ng/ml | 667 ng/ml |
| **0.02** | 66.7 ng/ml | 133.4 ng/ml |
| **0.01** | 33.35 ng/ml | 66.7 ng/ml |

## 2.5 Cytotoxicity Assay Controls

Prior to beginning the decontamination efficacy testing, several control experiments were conducted. Controls tested were conducted in triplicate. The effect of different culture media was tested using the anthrax toxins to determine which culture medium was best for use in the assay. In the first experiment PBS, Opti-MEM and low serum DMEM were compared. Opti-MEM is a modified minimal essential medium (MEM) containing a reduced amount of serum and no phenol-red. The low serum DMEM used was the same DMEM complete described previously except with only 2% FBS. For these tests fresh stock LF and PA toxins were used (no soil load added or drying) and were diluted in the culture media being tested. The cytotoxicity assay was performed as described in the previous section. Following these experiments Opti-MEM was also compared to a low serum MEM containing no phenol red (Invitrogen) in the same manner.

For both ricin and the anthrax toxins, the effect of drying was tested to determine if the toxins retain toxicity after drying. MTT cytotoxicity assay results for fresh stock toxins were compared with those of dried toxins. The effect of soil load was also investigated to determine if the soil load interfered with or increased toxicity of ricin or anthrax lethal toxin. This was done by comparing the MTT cytotoxicity assay results of the toxins in the presence and absence of soil load. Neutralization controls were conducted on the 1% sodium pyruvate Opti-MEM neutralizing solution. MTT cytotoxicity assay results for both ricin and the anthrax toxins were compared for the tests outlined in Table 2.5.1 to ensure the neutralizer was not cytotoxic to the tissue culture cells, does not interfere with toxin activity and was capable of neutralizing the activity of GCD and VHP.

Cell viability data for each variable was calculated as described previously. Data was then analyzed using Microsoft Excel 2010 for statistically significant differences between variables. The percent viability of cell treated with different dilutions was calculated separately. In comparisons of two variables a two tail student's t-test assuming equal variance was used. In comparisons of more than two variables single factor ANOVA (analysis of variance) was used.

Table 2.5.1: Neutralization controls conducted for the cytotoxicity assay used for the detection of anthrax lethal toxin and ricin. Neutralizer used is 1% sodium pyruvate in Opti-MEM.

| Test | Toxin | Medium eluted in | Expected result | What is tested |
|---|---|---|---|---|
| **Positive Control** | Dried toxin | Opti-MEM | Cells non-viable | Indicates toxin is cytotoxic to cells |
| **Cytotoxicity Control** | No toxin | Neutralizer | Cells viable | Indicates neutralizer is not cytotoxic to cells |
| **Interference Control** | Dried toxin | Neutralizer | Cells non-viable | Indicates neutralizer does not interfere with toxin activity |
| **GCD Neutralization Control** | Dried Toxin | Neutralizer exposed to GCD | Cells non-viable | Indicates 1% sodium pyruvate neutralizes GCD |
| **VHP Neutralization Control** | Dried Toxin | Neutralizer exposed to VHP | Cells non-viable | Indicates 1% sodium pyruvate neutralizes VHP |

## 2.6 Detection of toxins by protein gel electrophoresis

Toxins exposed to GCD and VHP were also analyzed by SDS-PAGE for degradation using the NuPAGE® Electrophoresis System (Invitrogen) under non-reducing conditions. Samples were prepared by the addition of 6x sample buffer consisting of 4x Tris·Cl/SDS, glycerol, SDS, bromophenol blue and 2% β-Mercaptoethanol (1.5x final concentration) followed by denaturing at 80°C for ten minutes. Controls of stock toxin with and without soil load were also prepared similarly by diluting 10 µl of stock toxins in 500 µl of sterile water. 15 µl of each sample and 10 µl of Benchmark™ pre-stained protein ladder (Invitrogen) was loaded on NuPAGE® Novex® 4-12% Bis Tris pre-cast gels (Invitrogen). Electrophoresis was conducted using the XCell *Sure Lock*™ Minicell (Invitrogen) with NuPAGE® MES SDS running buffer (Invitrogen) at 120 volts for one hour according to manufacturer's instructions. Following electrophoresis the protein gels were stained for approximately 2 hours using an Express Coomassie stain consisting of 45% methanol, 10% glacial acetic acid, 45% water and 3 g/L Coomassie Brilliant Blue R250. To visualize the proteins the gels, were destained for 3 -18 hours with a Rapid destain consisting of 500 ml sterile water, 100 ml acetic acid and 400 ml methanol.

## 2.7 Ricin GFP protein inhibition assay

The GFP protein inhibition assay as first described by Halter et al. (2009) was investigated for its use in detecting ricin after exposure to GCD and VHP. The assay was designed as a direct way of detecting ricin activity by measuring the inhibition of synthesis of the GFP reporter caused by the enzymatic activity of ricin (Halter, Almeida et al. 2009). As summarized in Figure 2.7.1, a construct containing reporter gene, destabilized GFP, under the

control of an immediate early CMV promoter, and the antibiotic resistance marker for neomycin/kanamycin was created and transfected into Vero 76 cells. These GFP expressing cells can then be used for the protein inhibition assay by measuring the loss of cellular GFP due to inhibition of ribosome activity caused by ricin (Figure 2.7.2).

The CMV/pZGreen construct was created by ligating the CMV immediate early promoter to the GFP gene in the pZsGreen1-DR vector (Clonetech Laboratories Inc., Mountain View, CA, USA), using a protocol similar to that described in Halter et al. (2009). First the CMV IE promoter was amplified by PCR from the pQCXIP vector (Clonetech Laboratories Inc.) in a total volume of 50 µl using iproof High-Fidelity DNA polymerase (Bio-Rad Laboratories, Mississauga, Ont., Canada): 10 µl 5X iproof HF buffer (1X final concentration), 1 µl dNTP mixture (200µM each nucleotide final concentration), 0.5 µl 100 µM sense primer and 0.5 µl 100 µM anti-sense primer (1 µM final concentration each) (Appendix A.1), 1 µl iproof HF DNA polymerase (2 units/µl final concentration), 0.5 µl DNA template (pQCXIP vector), and 36.5 µl sterile water. The cycling conditions were as follows; initial denaturation period of 98°C for one minute, repeated cycles (28 cycles) of denaturation, hybridization and extension at 98°C for 20 seconds, 60°C for 30 seconds and 72°C for 30 seconds, respectively, with a final extension at 72°C for two minutes and a 4°C indefinite pause. The PCR product was then gel isolated and purified using the QIAquick gel extraction kit (QIAGEN Inc., Toronto, Ont., Canada) according to manufacturer's protocols. A 1% agarose gel was made using one gram agarose powder and 100 ml of 1X TBE buffer. The PCR reaction and 1kb+ DNA ladder (New England BioLabs, Pickering, Ont., Canada) mixed with 1x loading dye (SDS, ficoll, EDTA and bromophenol blue) were run at 120 volts for 30 minutes.

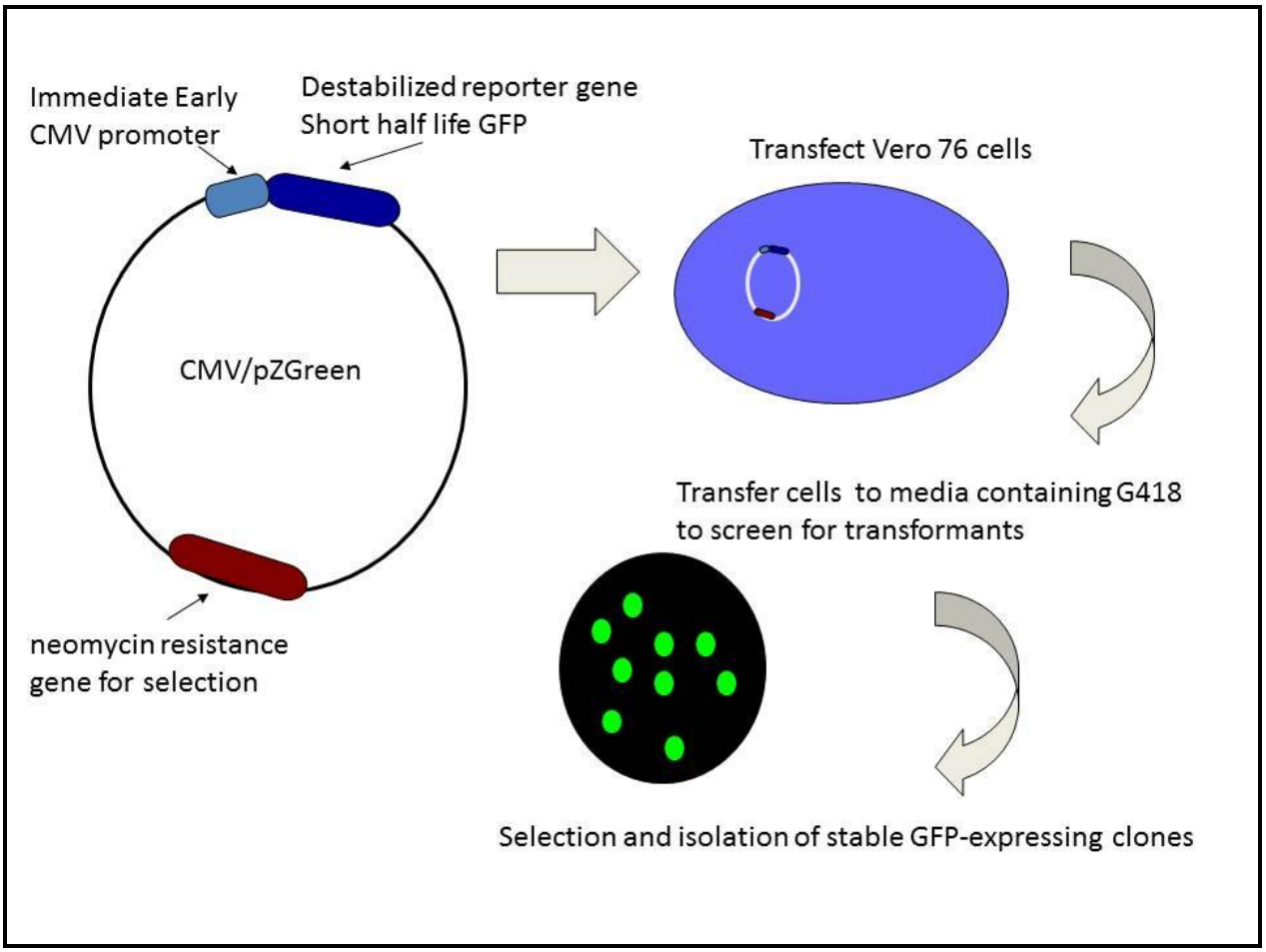



Figure 2.7.1: Generation of the GFP expressing Vero 76 cells.

CMV/pZGreen construct containing destabilized GFP under immediate early CMV promoter is created and transfected in Vero 76 cells. G418 compound is used to screen GFP expressing and neomycin resistant clones, followed by the isolation of a stable GFP expressing clone.

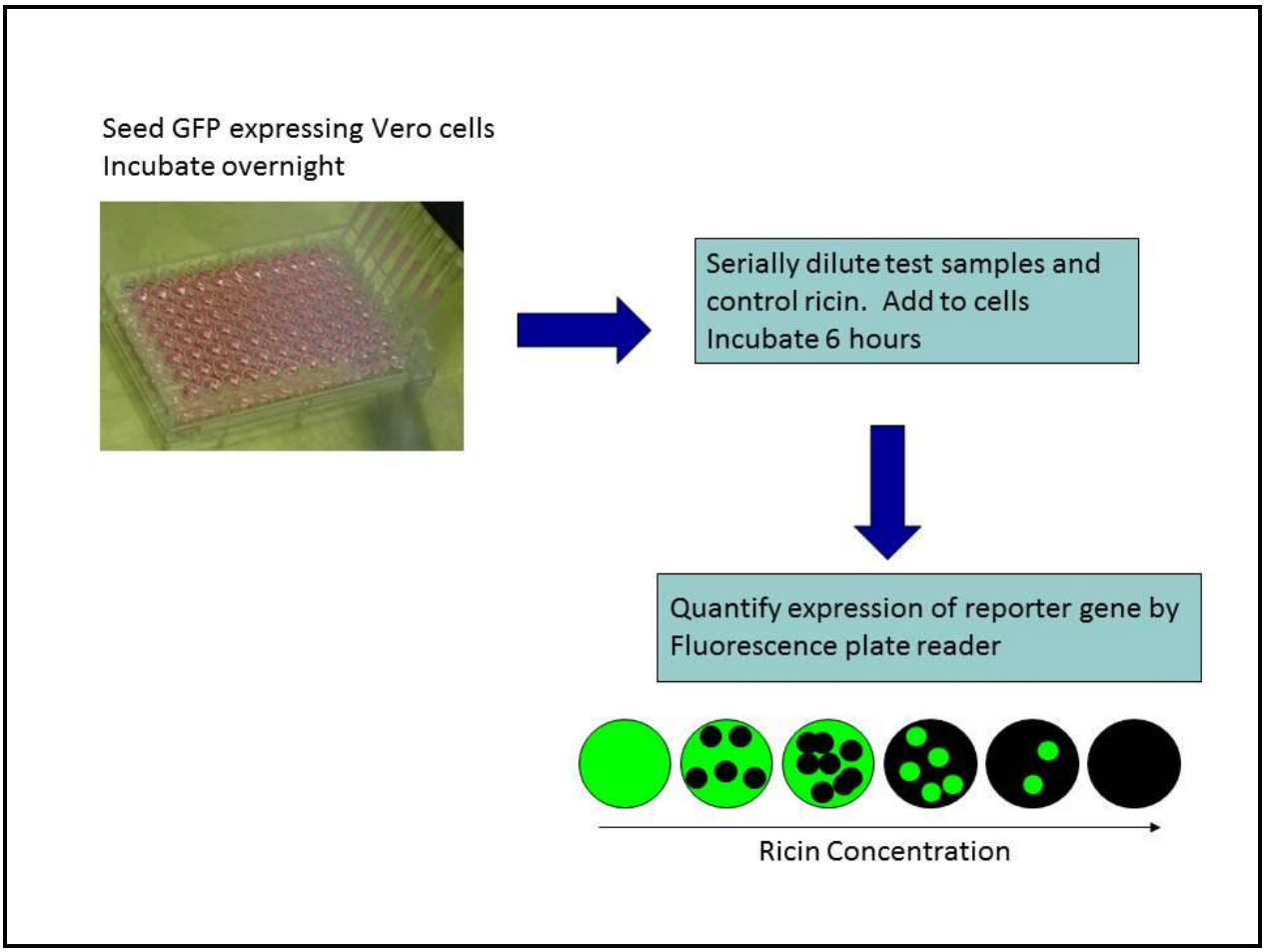



Figure 2.7.2: Ricin GFP protein inhibition assay

96 well tissue culture containing GFP expressing Vero cells are prepared. The following day cells are treated with dilutions of ricin test and controls, ending with the quantification of reporter gene.

The CMV IE promoter PCR product and GFP vector pZsGreen1-DR were digested with *Bam*HI and *Xho*I (New England BioLabs) restriction enzymes according to reactions in Appendix A.2 and were incubated at 37°C for one hour. The digested CMV IE promoter PCR product was purified using a Kleenspin column (BioRad Laboratories) and the digested pZsGreen1-DR vector was gel isolated and purified using the QIAquick gel extraction kit (QIAGEN Inc.). The final CMV/pZGreen construct was created by ligation of the CMV IE promoter PCR product (insert) to the pZsGreen1-DR vector. The ligation reaction was: 2 µl 10X T4 DNA ligase buffer (New England BioLabs), 5 µl digested vector, 12 µl digested insert and 1 µl T4 DNA ligase (New England BioLabs), and conducted at 18°C for four hours.

The CMV/pZGreen construct was then transformed by chemical methods with Chemically-Competent Top10 *E.coli* (Invitrogen). Transformation was conducted using the manufacturer's protocol: thawing the chemically-competent cells from -70°C on ice, adding 3 µl of the CMV/pZGreen construct to the completely thawed competent cells by gentle mixing and incubating for 30 minutes on ice. The competent cells were then heat shocked at 42°C for 30 seconds and immediately transferred to ice before adding 250 µl of SOC medium. Cells were allowed to recover by shaking at 280 rpm at 37°C for one hour before spread plating the mixture on LB (Luria-Bertani) medium plates supplemented with 100 µg/ml of kanamycin and incubating at 37°C overnight. The resulting transformants were then subjected to PCR screening. Reactions for PCR screening were 30 µl total volume and contained Go *Taq* green master mix (ProMega, Nepean, Ont., Canada), 250nM of the sense and antisense primer (Appendix A.1) and a single colony of which the residual cells on the loop were streaked onto an LB-kanamycin plate for future harvesting of positive clones. The PCR cycling conditions were as follows: initial denaturation period of 95°C for 10 minutes, repeated cycles (40 cycles) of

denaturation, hybridization and extension at 95°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds, respectively, with a final extension at 72°C for five minutes and a 4°C indefinite pause.

Positive clones were then grown in 5 ml of LB broth containing kanamycin and the plasmids were isolated using QIAprep spin miniprep kits (QIAGEN Inc.) according to manufacturer's protocols. These isolated plasmids were then confirmed by restriction digest as outlined in Appendix A.2 and incubated at 37°C for one hour. The positive clone 21 chosen for further work was grown in 100 ml LB-kanamycin broth and the plasmid was isolated using a QIAGEN plasmid purification maxiprep kit (QIAGEN Inc.). This positive clone was then DNA sequenced by the NML DNA core facility using the primers outlined in Appendix A.1.

The maxiprep isolated plasmid of clone 21 was then used to transfect Vero 76 cells using the Attractene lipotransfection reagent (QIAGEN Inc.). Transfection reactions were set up in 6 well tissue culture plates containing Vero 76 cells at ~80% confluency. According to the manufacturer's protocols, 4.5 µl of the Attractene Transfection reagent was added to 100 µl of the plasmid DNA and incubated at room temperature for 15 minutes. Culture media was removed from the cells and replaced with fresh complete DMEM. The transfection complexes were added dropwise to the cells with gentle swirling of the plate to ensure even distribution. Plates were then incubated at 37°C overnight. On the next day, the culture medium was removed from the cells and replaced with fresh medium containing 800 µg/ml of the selecting agent G418 (Invitrogen).

Attempts were made to isolate a single stably expressing GFP clone by dilution method. Transfected cells were removed by trypsinization and serially diluted in DMEM complete culture

medium containing the selecting agent G418 and transferred to a 48 well plate. Plates were monitored for wells containing stably expressing GFP cells resulting from a single cell, which could then be further propagated.

# CHAPTER 3.0: RESULTS

## 3.1 Effect of Medium on the MTT Cytotoxicity Assay

In the first experiment to determine the best culture medium for use in the cytotoxicity assay, PBS, low serum DMEM and Opti-MEM were compared. When testing PBS, cells treated with PBS only were found to have very low absorbance values indicating the cells were non-viable. Based on this data PBS was not investigated for further use. In tests using the DMEM with 2% serum, absorbance values of the negative control (no toxin added) were relatively high indicating viable cells; cells exposed to lower dilutions of lethal toxin exhibited a decrease in cell viability (Figure 3.1.1). In tests using Opti-MEM, absorbance values of the negative control (no toxin added) were relatively high indicating viable cells; cells exposed to lower dilutions of lethal toxin exhibited a large decrease in cell viability (Figure 3.1.1). Based on results shown in Figure 3.1.1, the phenol red free Opti-MEM was chosen for further study as anthrax lethal toxin exhibited the greatest toxicity in this medium. Opti-MEM was further compared to the phenol red free, low serum MEM. As seen in Figure 3.1.2, all dilutions of lethal toxin in MEM were non-toxic, as indicated by the high levels of cell viability. Based on these results Opti-MEM was used of for all further cytotoxicity testing.

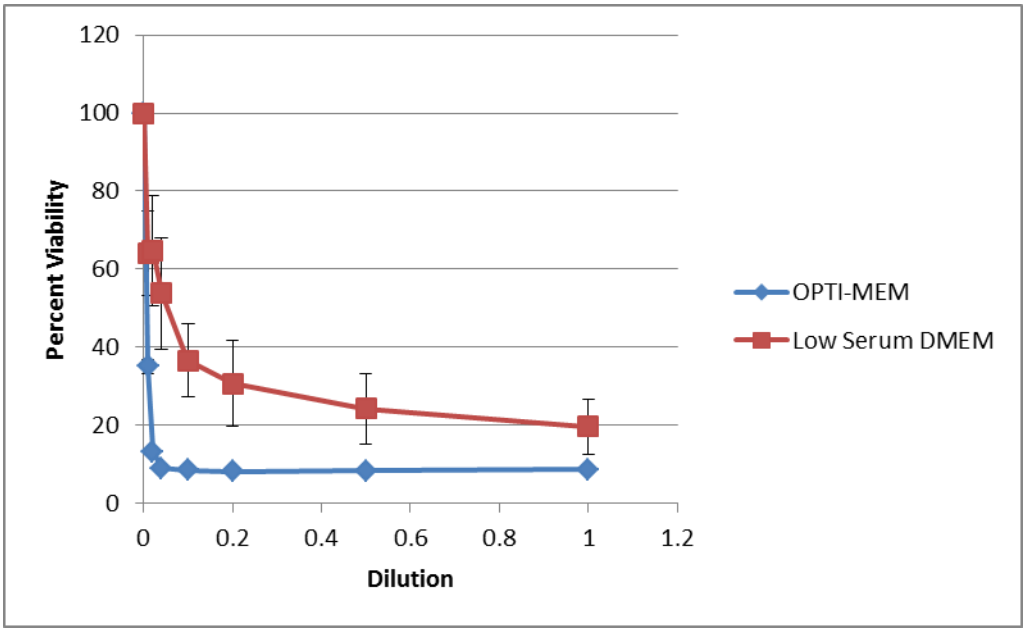



Figure 3.1.1: Effect of low serum DMEM and Opti-MEM on MTT cytotoxicity assay.

Percent viability of cells treated with dilutions of anthrax lethal toxin in low serum DMEM and Opti-MEM. Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

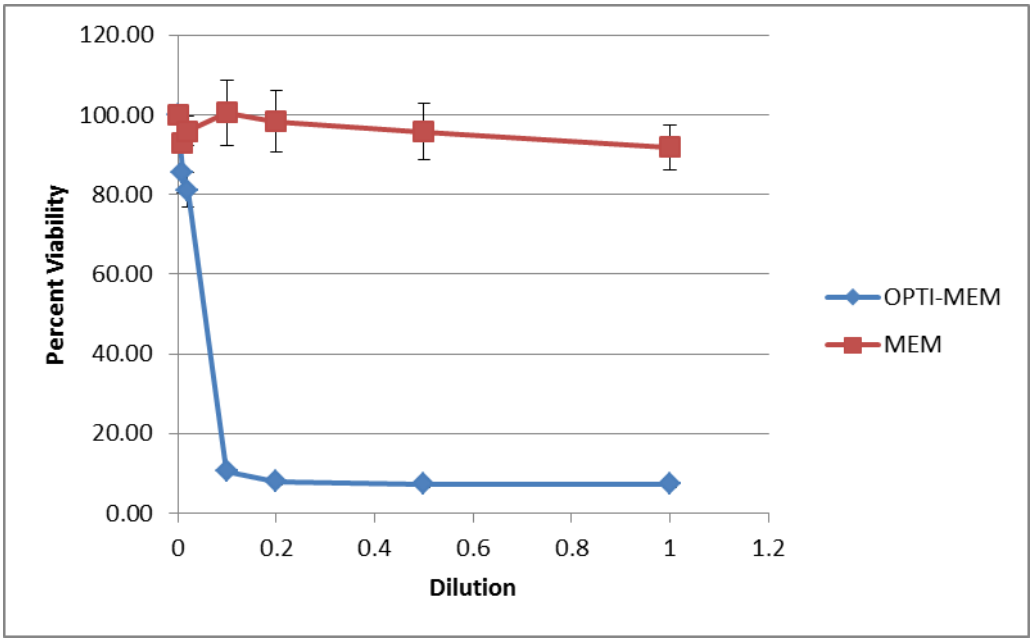



Figure 3.1.2: Effect of MEM and Opti-MEM on MTT cytotoxicity assay.

Percent viability of cells treated with dilutions of anthrax lethal toxin in phenol red free MEM and Opti-MEM. Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

## 3.2 Anthrax Toxin MTT Cytotoxicity Assay Controls

As seen in Figure 3.2.1, most dilutions of anthrax lethal toxin exhibited similar levels of toxicity for dried and not dried toxin, as well as with and without soil load. At higher dilutions the presence of the soil load resulted in lower levels of cell viability (higher toxicity) in both dried and fresh toxin. Little difference was seen between fresh and dried toxin. Importantly no difference was evident between fresh/dry or soil load/no soil load for the undiluted toxin. The data shown in Figure 3.2.1 indicate drying the toxin and the addition of the soil load does not decrease its toxicity. Based on these data, all further anthrax toxin experiments were conducted with dried toxin and soil load.

Neutralization controls outlined in Table 2.5.1 were conducted using the anthrax lethal toxin to ensure 1% sodium pyruvate was an appropriate neutralizer for VHP and GCD and could be used in the MTT cytotoxicity assay. As seen in Figure 3.2.2, similar levels of cell viability were observed for the positive control and interference control, indicating the neutralizer does not interfere with lethal toxin activity or the MTT cytotoxicity assay. Slightly lower levels of cell viability were observed in the undiluted cytotoxicity control (Figure 3.2.2). High levels of variation were observed between replicates at this dilution. Cell viability of neutralizer exposed to VHP and GCD was compared to unexposed neutralizer showing very little difference between the tests (Figure 3.2.3). These results indicate 1% sodium pyruvate Opti-MEM does neutralize GCD and VHP so that lethal toxin added to this solution does not result in a decrease of toxicity. Based on the data in Figures 3.2.2 and 3.2.3, 1% sodium pyruvate was used as the neutralizer for VHP and GCD.

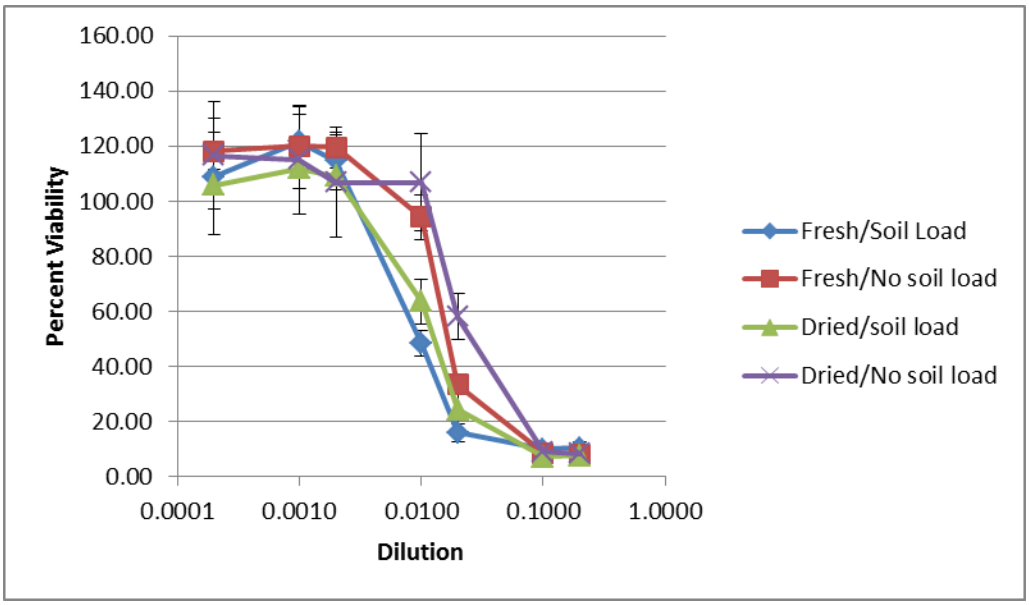



Figure 3.2.1: Effect of drying and soil load on lethal toxin activity.

Percent viability of cells treated with dilutions of lethal toxin dried and not dried, with and without soil load. Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

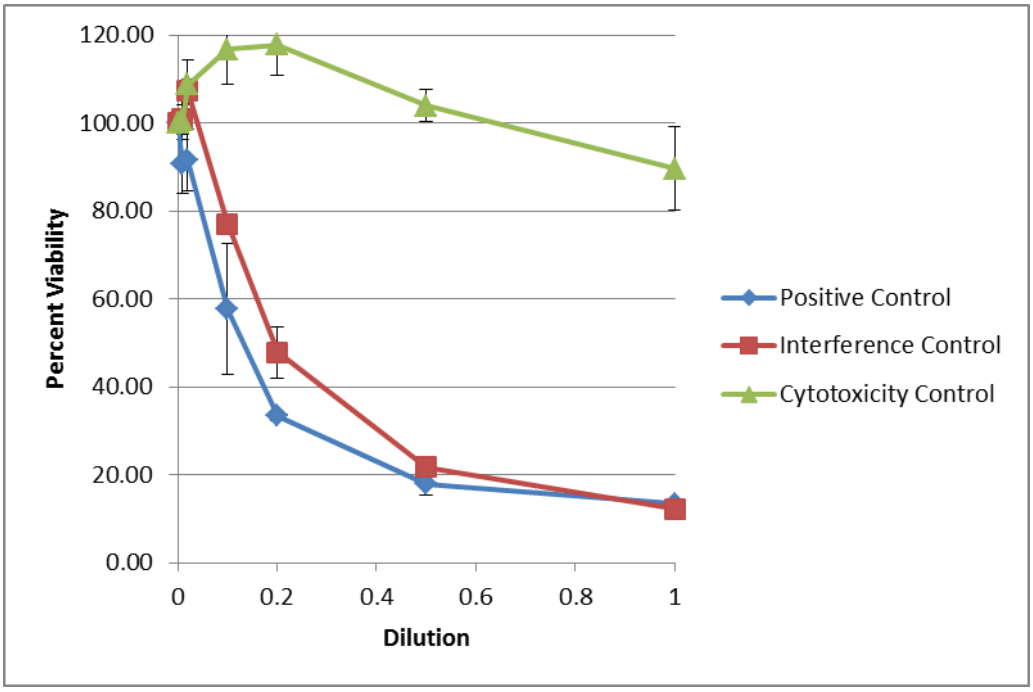



Figure 3.2.2: Neutralization controls for 1% sodium pyruvate Opti-MEM for the anthrax toxin cytotoxicity assay.

Positive control is cells treated with dilutions of lethal toxin in Opti-MEM, interference control is cells treated with dilutions of lethal toxin in neutralizer and cytotoxicity control is cells treated with neutralizer containing no toxin. Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

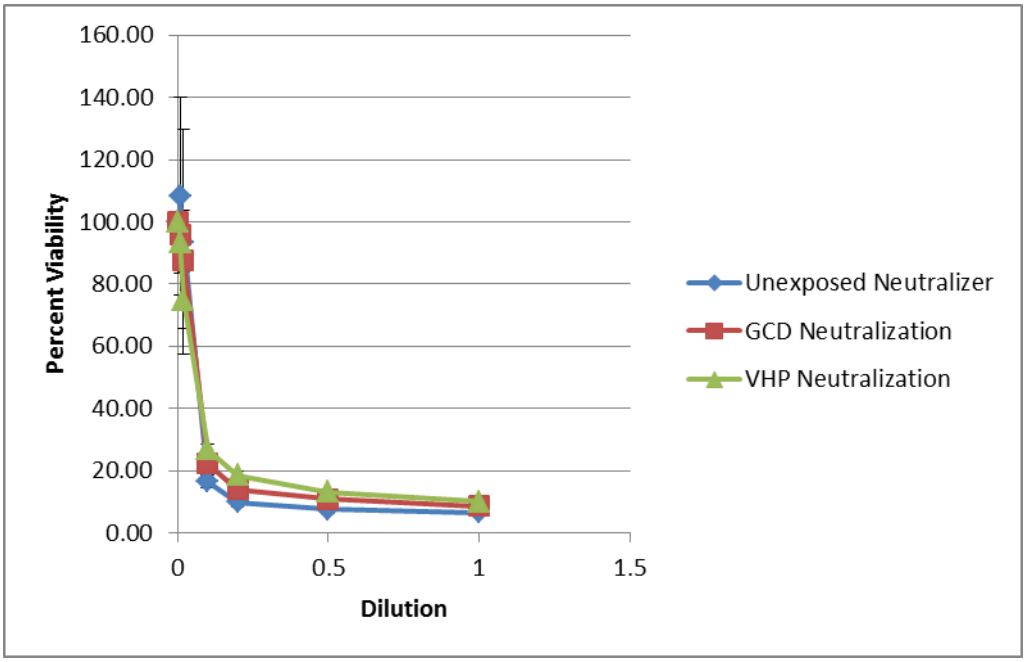



Figure 3.2.3: GCD and VHP neutralization controls using 1% sodium pyruvate Opti-MEM for the anthrax toxin cytotoxicity assay.

Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

## 3.3 Anthrax toxins and VHP: MTT Cytotoxicity Assay

Protective antigen was exposed to approximately 800-1000ppm of VHP for 1, 5 and 10 minutes as well as 4 hours. Figure 3.3.1 illustrates the percent viability of cells treated with dilutions of lethal toxin containing the exposed PA in comparison to the unexposed control. PA exposed to VHP for 1 minute resulted in cell viabilities similar to those of the unexposed control. Longer exposures of 5 and 10 minutes resulted in increases in cell viability (indicative of a decrease in toxicity) for all dilutions of lethal toxin containing the exposed PA including the undiluted toxin. Further exposure of 4 hours resulted in close to 100% cell viability for all dilutions.

Lethal factor was exposed to VHP under the same conditions as PA. Cell viability data are presented in Figure 3.3.2 and indicates similar results to PA exposed to VHP. Exposure to VHP for 1 minute resulted in similar cell viabilities to the unexposed control and further exposures of 5 and 10 minutes resulted in increased cell viability at all dilutions. Four hours of VHP exposure resulted in cell viabilities of 100% at all dilutions. A summary of the cell viability values of only the undiluted LF and PA exposed to VHP (Figure 3.3.3) shows a decrease in the toxicity of both LF and PA when exposed for at least 5 minutes. After 4 hours of VHP exposure the toxicity of both LF and PA was decreased so that cells treated with these toxins were 80-100% viable, with those treated with the exposed LF showing the highest levels of viability.

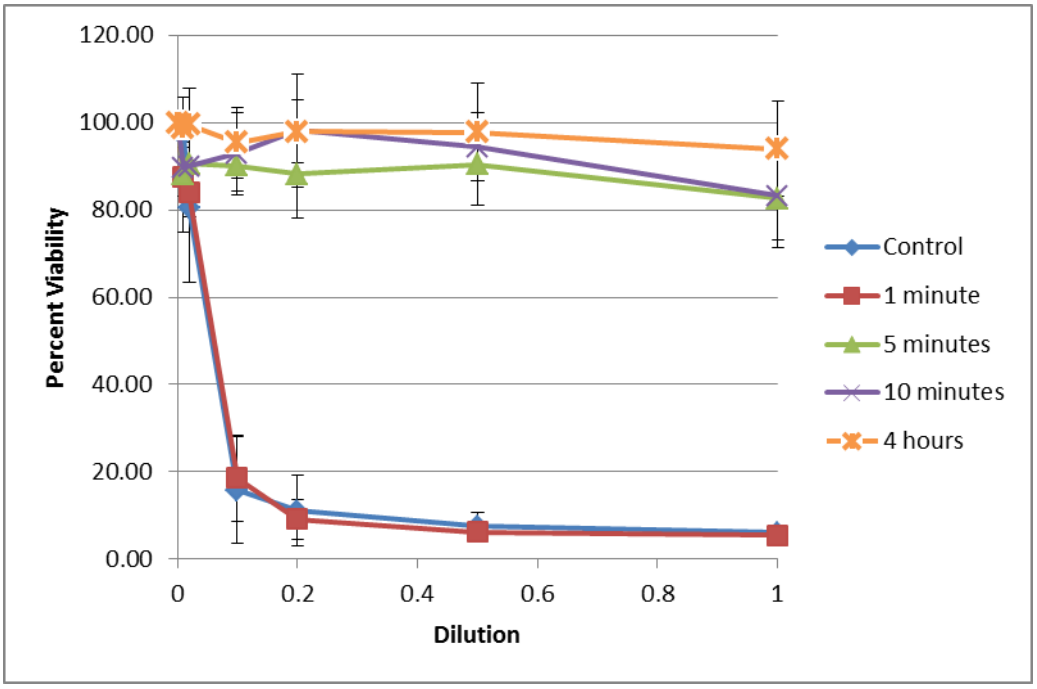



Figure 3.3.1: Protective Antigen exposed to VHP.

Percent viability of cells treated with dilutions of lethal toxin containing PA exposed to VHP for 1, 5 and 10 minutes, and 4 hours. Unexposed PA is used as the control. Undiluted toxin is expressed as 1. Nine replicates of the controls and six replicates of each time point were conducted.

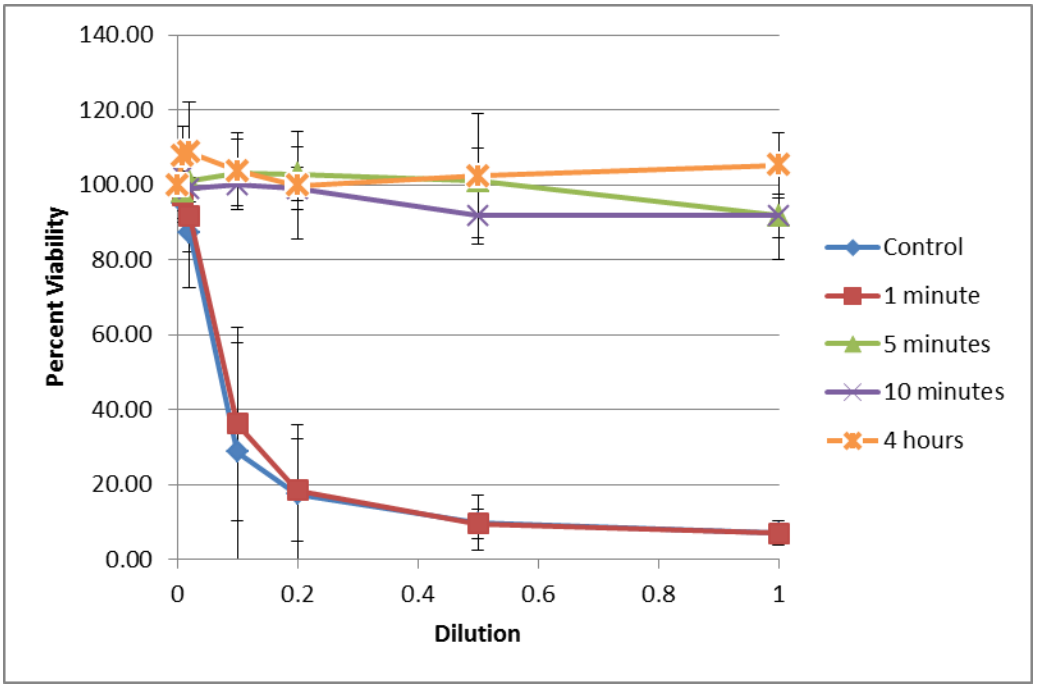



Figure 3.3.2: Lethal Factor exposed to VHP.

Percent viability of cells treated with dilutions of lethal toxin containing LF exposed to VHP for 1, 5 and 10 minutes, and 4 hours. Unexposed LF is used as the control. Undiluted toxin is expressed as 1. Nine replicates of the controls and six replicates of each time point were conducted.

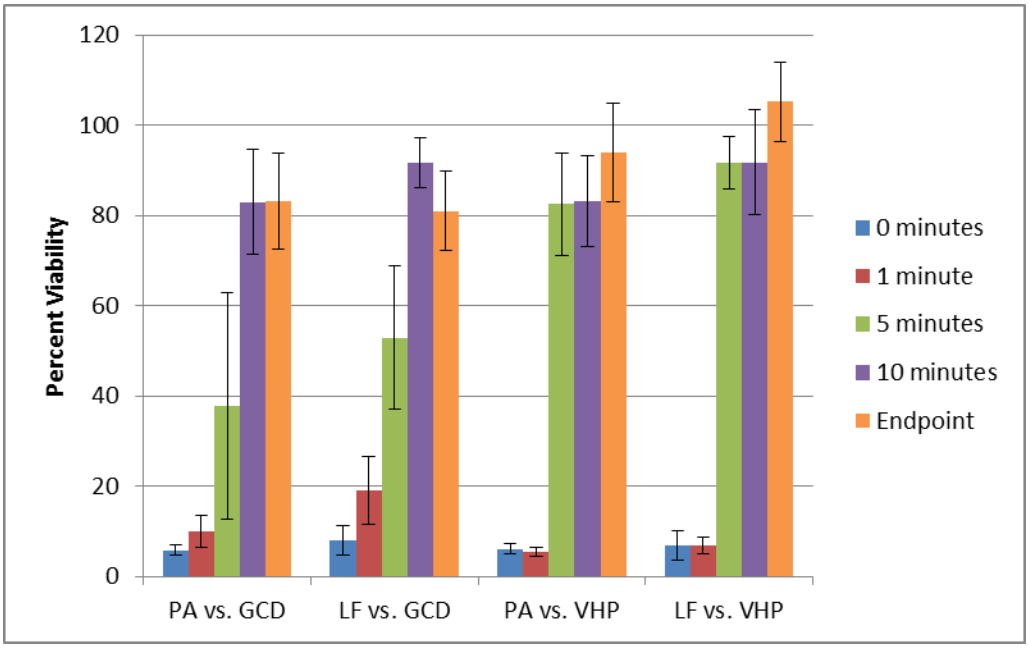



Figure 3.3.3: Summary of LF and PA exposure to VHP and GCD.

Percent viability of cells treated with undiluted lethal toxin containing either LF or PA exposed to VHP or GCD. Endpoint exposure times were 2 hours for GCD and 4 hours for VHP.

## 3.4 Anthrax Toxins and GCD: MTT Cytotoxicity Assay

Protective antigen was exposed to 5mg/L of GCD for 1, 5 and 10 minutes as well as for 2 hours. As with exposure to VHP, PA exposed to GCD for 1 minute resulted in similar levels of cell viability as the unexposed control (Figure 3.4.1). Exposures of 5 minutes caused a relatively small increase in cell viability, with the largest increases in the higher dilutions of toxin. Considerable variability was observed between the 5 minute replicates (Figure 3.4.1). Additional exposures of 10 minutes and 2 hours lead to improved cell viability at all dilutions with similar levels between the two time points. The summary in Figure 3.3.3 reveals a greater level of viability in cells treated with PA exposed to VHP than those treated with GCD exposed PA, especially in the 5 minute time point.

Lethal factor was exposed to the GCD under the same conditions and for the same time points. As seen in Figure 3. 4.2, GCD for 5 minutes also appeared to have a poorer effect on the toxicity of LF, with similar cell viability levels as those resulting from PA exposed to GCD and high levels of variability between replicates. As with all other anthrax toxin tests, exposures of 1 minute resulted in little change in cell viability compared to the unexposed controls. Additional exposures of 10 minutes and 2 hours caused higher levels in cell viability although the lengthiest of the exposure did not result in an improvement beyond the viability observed at 10 minutes (Figure 3.4.2). In the cells treated with undiluted exposed LF the maximum level of viable cells was seen at the 10 minute time point with a slight drop in viability at the 2 hour exposure (Figure 3.3.3).

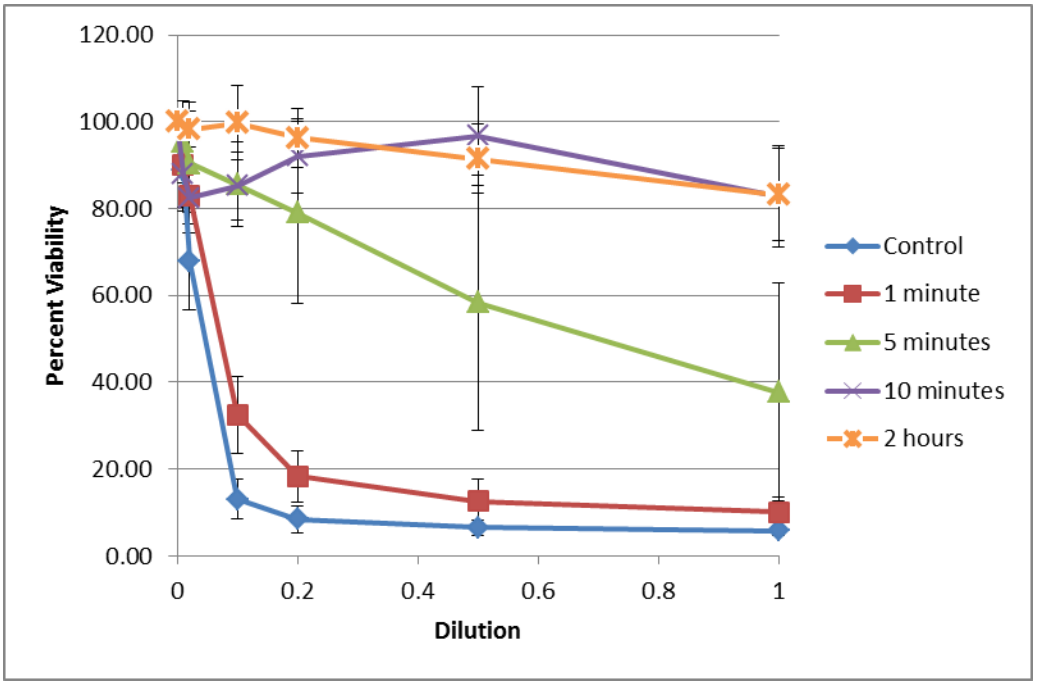



Figure 3.4.1: Protective Antigen exposed to GCD.

Percent viability of cells treated with dilutions of lethal toxin containing PA exposed to GCD for 1, 5 and 10 minutes, and 2 hours. Unexposed PA is used as the control. Undiluted toxin is expressed as 1. Nine replicates of the controls and six replicates of each time point were conducted.

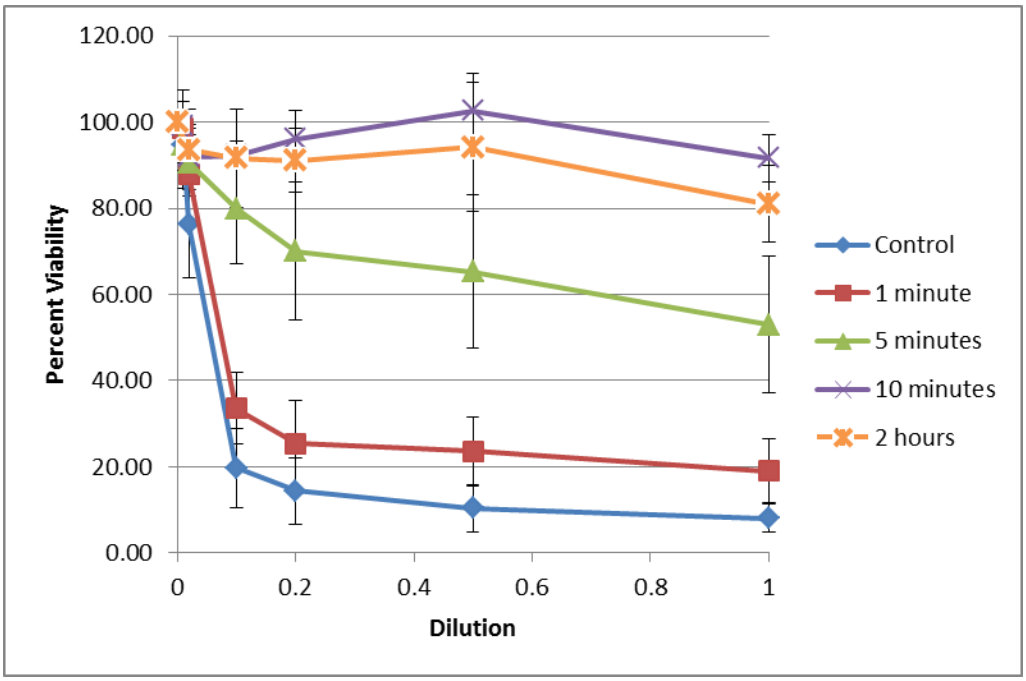



Figure 3.4.2: Lethal Factor exposed to GCD.

Percent viability of cells treated with dilutions of lethal toxin containing LF exposed to GCD for 1, 5 and 10 minutes, and 2 hours. Unexposed LF is used as the control. Undiluted toxin is expressed as 1. Nine replicates of the controls and six replicates of each time point were conducted.

## 3.5 Ricin MTT Cytotoxicity Assay Controls

As with the anthrax toxins, the effect of drying and the addition of soil load on the toxicity of ricin in the MTT cytotoxicity assay were tested. As seen in Figure 3.5.1, there was no difference in the viability of cells treated with fresh or dried toxin. The presence of soil load also resulted in no change in the toxicity of ricin. All further tests with ricin were conducted using dried toxin and with the addition of the soil load.

Opti-MEM containing 1% sodium pyruvate was tested for its use as a neutralizing solution for VHP and GCD using the controls outlined in Table 2.5.1. The neutralizer was not found to interfere with ricin activity or the MTT cytotoxicity assay as the interference control exhibited similar results in cell viability as the positive control (Figure 3.5.2). The neutralizer did not appear to cause cytotoxicity except with the undiluted toxin where a slight drop in cell viability to approximately 95% was observed (Figure 3.5.2). Cell viability of neutralizer exposed to VHP and GCD was compared to unexposed neutralizer showing very little difference between the tests (Figures 3.5.3). These results indicate 1% sodium pyruvate Opti-MEM does neutralize GCD and VHP so that lethal toxin added to this solution does not result in a decrease of toxicity. Based on this data, 1% sodium pyruvate was used as the neutralizer for VHP and GCD for experiments in the detoxification of ricin.

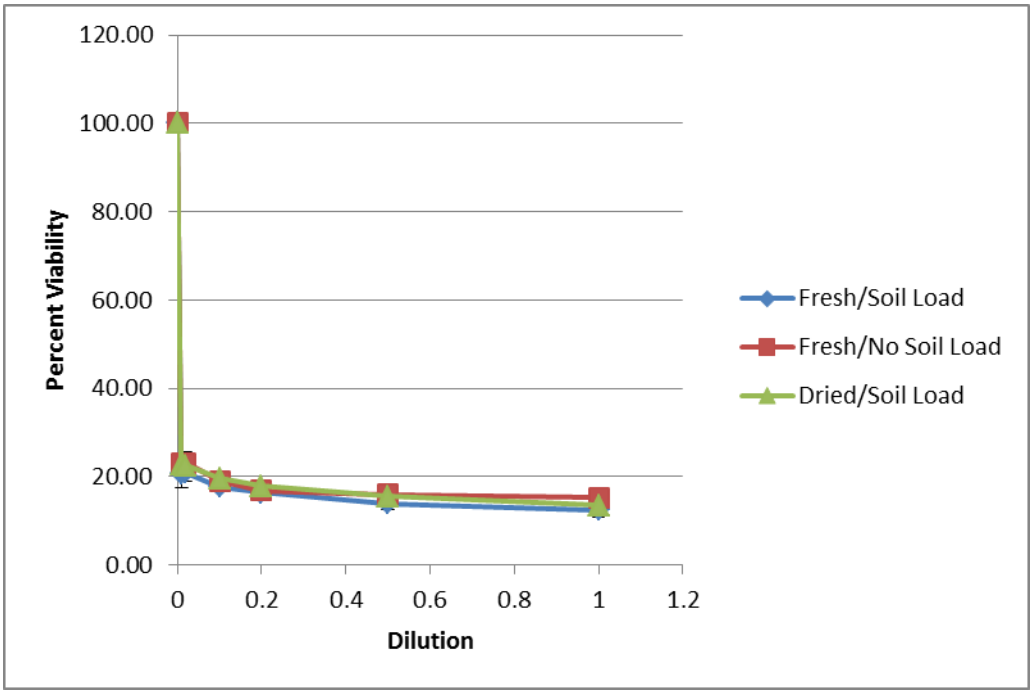



Figure 3.5.1: Effect of drying and soil load on ricin toxin activity.

Percent viability of cells treated with dilutions of ricin dried and not dried, with and without soil load. Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

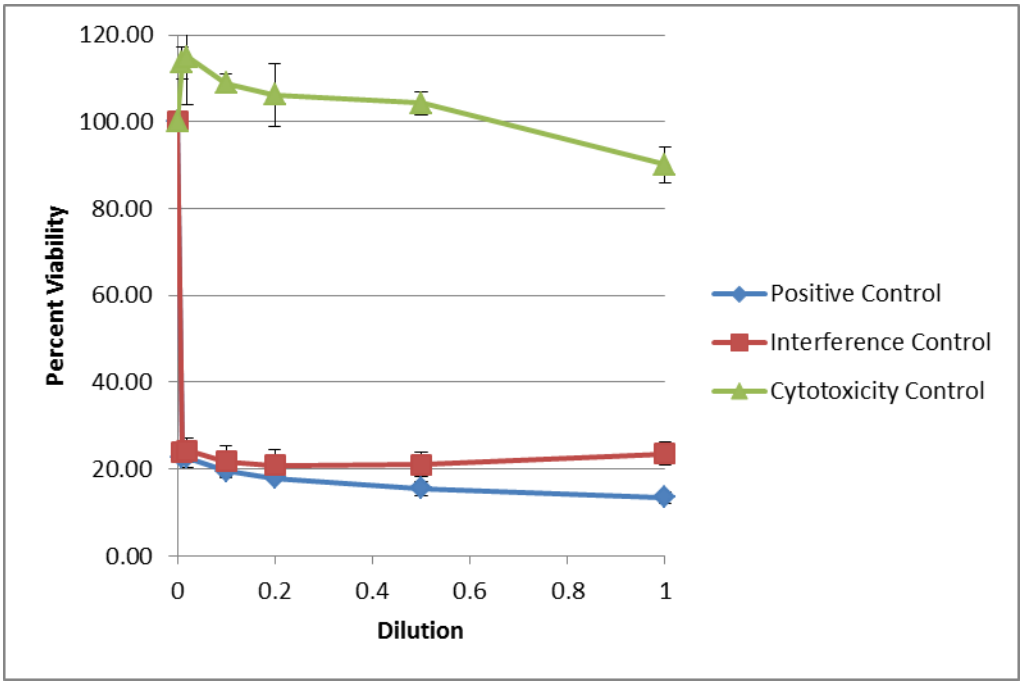



Figure 3.5.2: Neutralization controls for 1% sodium pyruvate Opti-MEM for the ricin toxin cytotoxicity assay.

Positive control is cells treated with dilutions of ricin in Opti-MEM, interference control is cells treated with dilutions of ricin in neutralizer and cytotoxicity control is cells treated with neutralizer containing no toxin. Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

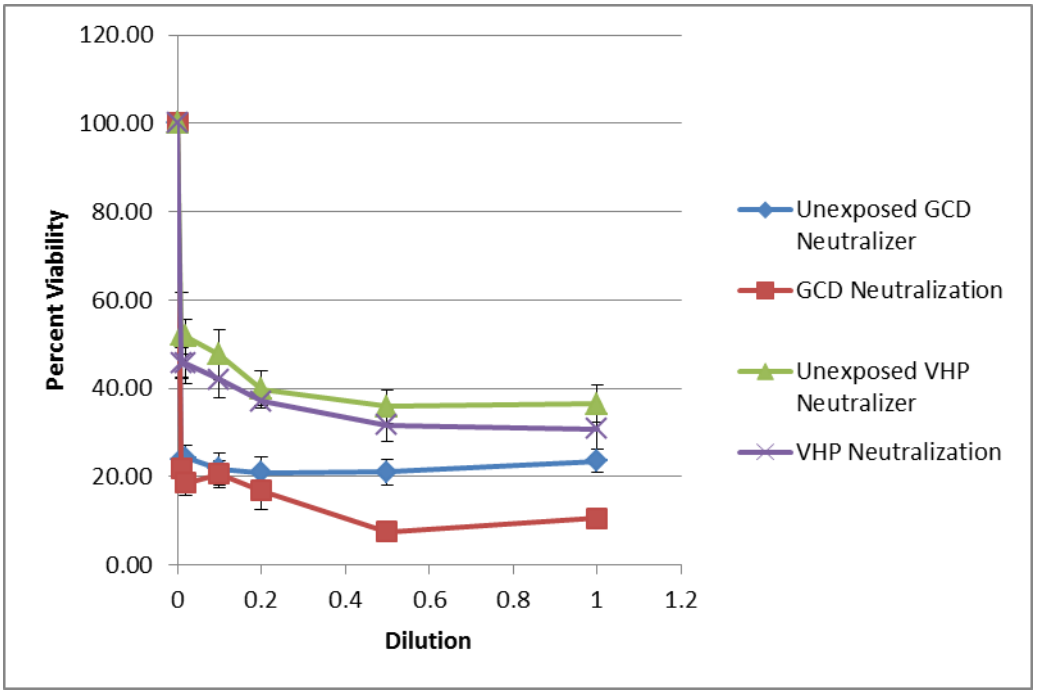



Figure 3.5.3: GCD and VHP neutralization controls using 1% sodium pyruvate Opti-MEM for the ricin toxin cytotoxicity assay.

As VHP and GCD exposures were conducted separately, separate controls were also conducted. Undiluted toxin is expressed as 1. Tests were conducted in triplicate.

## 3.6 Ricin and GCD: MTT Cytotoxicity Assay

Ricin was exposed to 5mg/ml of GCD for 1, 5, 10 and 30 minutes as well as for 2 hours. The 30 minute time point was added after preliminary tests showed little reduction in the toxicity of ricin after GCD exposure. As seen in Figure 3.6.1, GCD exposures of 1 and 5 minutes resulted in similar levels of viable cells as the unexposed control at all dilutions. At the 10 minute time point an increase in viable cells was observed in the higher dilutions although in the undiluted sample only a small increase was seen. Lengthier exposures of 10 minutes and 2 hours caused further increases in the levels of viable cells especially in the higher dilutions. The highest level of viable cells was found after 2 hours of exposure to GCD, with close to 100% viability seen in all dilutions except the undiluted where approximately 80% of cells were viable. The summary of undiluted tests in Figure 3.6.2 indicates a steady increase in viable cells in correlation with an increase in the length of GCD exposure, with largest levels of variation at the 10 and 30 minute time points.

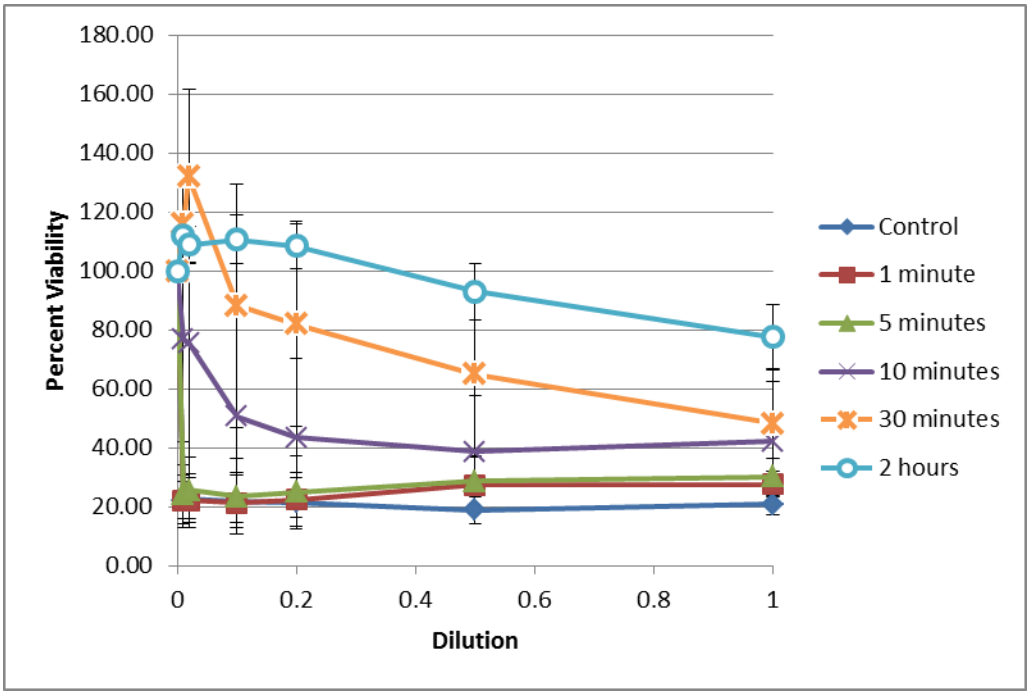



Figure 3.6.1: Ricin exposed to GCD.

Percent viability of cells treated with dilutions of ricin exposed to GCD for 1, 5, 10 and 30 minutes, and 2 hours. Unexposed ricin is used as the control. Undiluted toxin is expressed as 1. Nine replicates of the controls and six replicates of each time point were conducted.

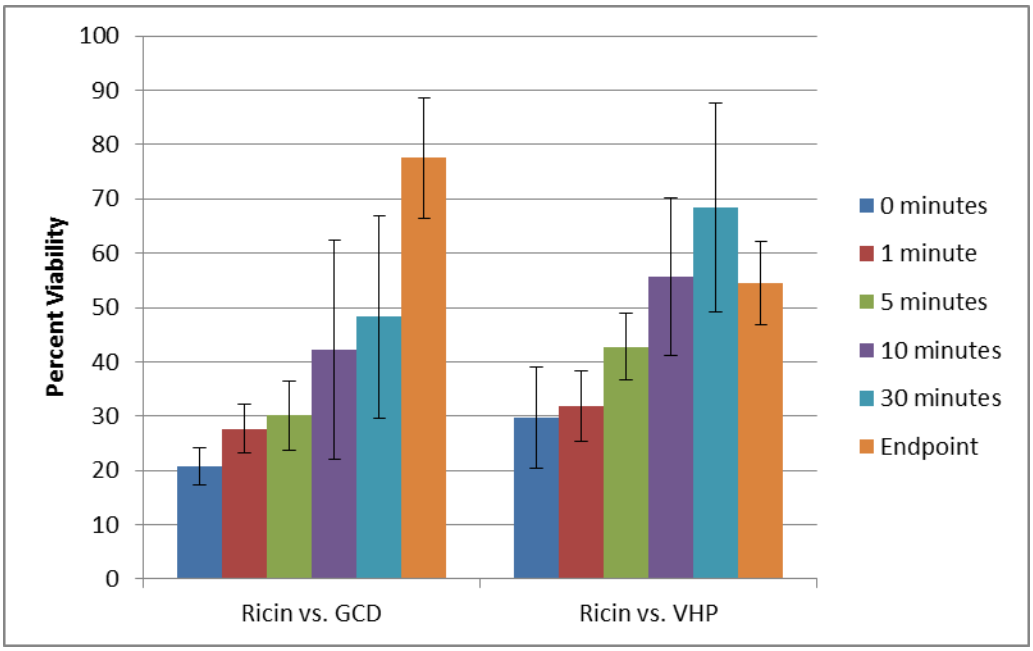



Figure 3.6.2: Summary of ricin exposure to VHP and GCD.

Percent viability of cells treated with undiluted ricin exposed to VHP or GCD. Endpoint exposure times were 2 hours for GCD and 4 hours for VHP.

## 3.7 Ricin and VHP: MTT Cytotoxicity Assay

Ricin was exposed to approximately 800-1000 ppm of VHP for 1, 5, 10 and 30 minutes as well a final time point of 4 hours. The 30 minute time point was added after preliminary tests showed little reduction in the toxicity of ricin after VHP exposure. As with GCD, shorter exposures to VHP resulted in cell viabilities similar to those of the unexposed control (Figure 3.7.1). Longer exposures of 10 and 30 minutes resulted in increased levels of viable cells up to approximately 70% with high variability. Further exposures of 4 hours however did not cause additional increases in cell viability. As seen in Figure 3.7.1, a drop in viable cells was seen to levels similar to those seen after 10 minutes VHP exposure. Indeed, the summary in figure 3.6.2 illustrates this increase in viable cells treated with toxin exposed to VHP for up to 30 minutes followed by a drop in cell viability for the 4 hour time point.

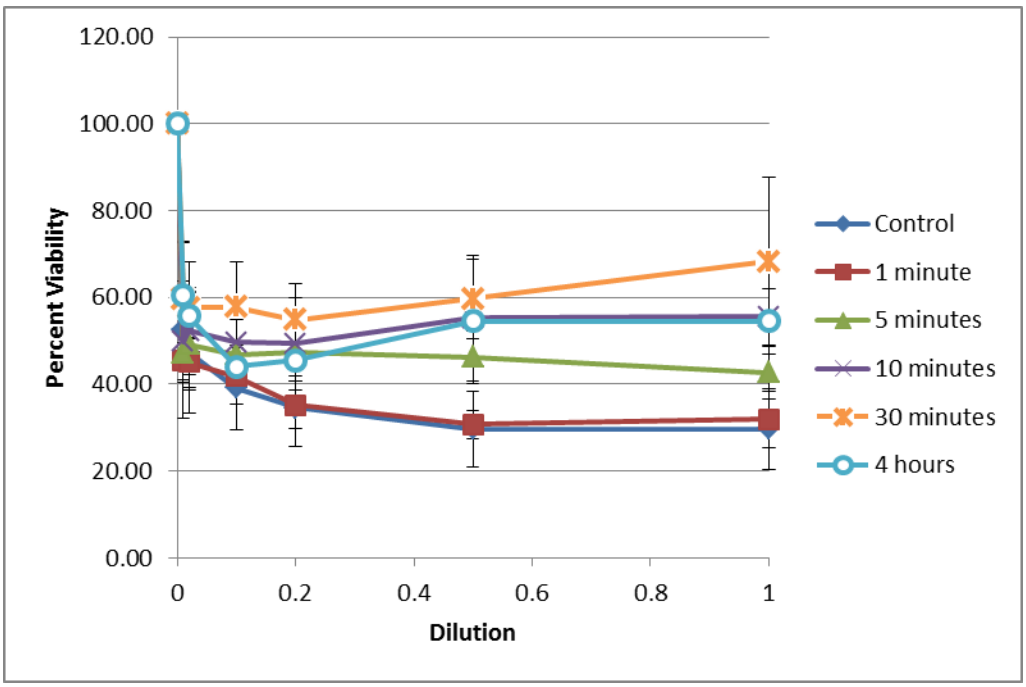



Figure 3.7.1: Ricin exposed to VHP.

Percent viability of cells treated with dilutions of ricin exposed to VHP for 1, 5, 10 and 30 minutes, and 4 hours. Unexposed ricin is used as the control. Undiluted toxin is expressed as 1. Nine replicates of the controls and six replicates of each time point were conducted.

## 3.8 Protein Gel Electrophoresis of Exposed Toxins

Exposed toxins eluted in sterile water were analyzed by gel electrophoresis along with controls for indication of protein degradation caused by GCD and VHP. The stock LF (no soil load and not dried) is seen as a single band at approximately 115-kDa (lane 2 of Figure 3.8.1A and Figure 3.8.2). With the addition of soil load more bands are seen representing the soil load proteins, which are also seen in the unexposed dried controls. As seen in lane 5 of Figure 3.8.1A, after only 1 minute of GCD exposure the band representing LF is not visible and only a smear of protein is visible. For exposures of 5 minutes and greater no protein is detected by gel electrophoresis. A faint band representing LF is still detected in the sample exposed to VHP for 1 minute (lane 5 of Figure 3.8.2). The band representing LF became fainter and the proteins in the sample became more smeared in VHP exposures of 5 and 10 minutes, while no protein was detected in the 4 hour exposure.

Similar results were observed for PA exposed to GCD and VHP. The single band for purified stock PA was approximately 115-kDa (lane 2 of Figure 3.8.1B and lane 10 Figure 3.8.2); extra bands were visible in the stock PA due to overflow from the adjacent well (Figure 3.8.1B). As with LF, additional bands were detected with the addition of soil load to PA (lanes 3 and 4 Figure 3.8.1B and lanes 11 and 12 Figure 3.8.2). A faint band of PA was detected in the sample expose to GCD for 1 minute and no protein was detected in samples of longer exposure (Figure 3.8.1B). Exposure to VHP resulted in fainter PA bands detected with lengthier exposures ending with no protein detected at the final time point of 4 hours, similar to the results observed for VHP exposed LF.

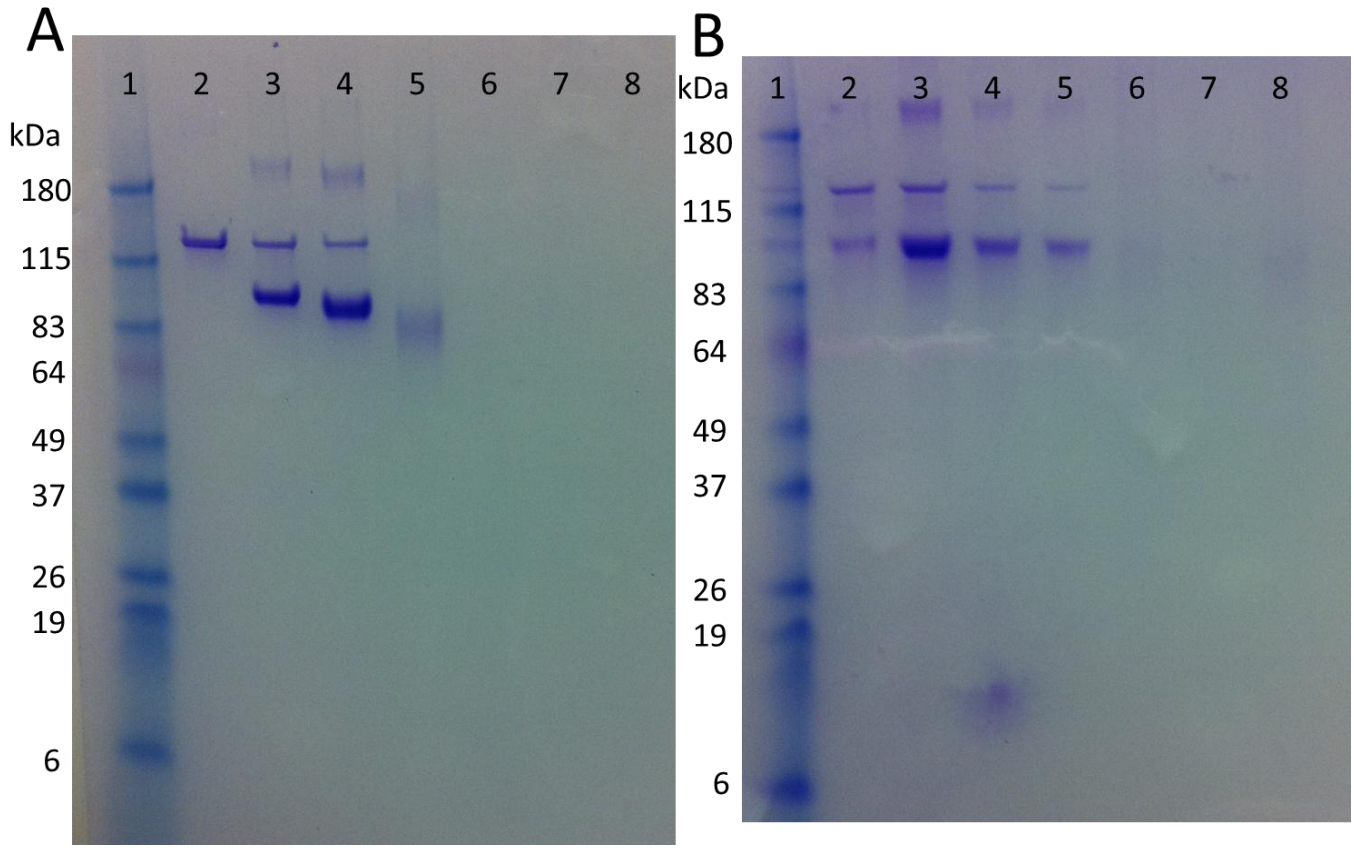



Figure 3.8.1: Protein gel electrophoresis of LF and PA exposed to GCD.

(A) LF exposed GCD: Lane 1: Benchmark™ pre-stained protein ladder (Invitrogen), Lane 2: stock LF, Lane 3: stock LF with soil load, Lane 4: unexposed control, Lane 5: 1 minute exposure, Lane 6: 5 minute exposure, Lane 7: 10 minute exposure, Lane 8: 2 hours exposure.

(B) PA exposed to GCD: Lane 1: Benchmark™ pre-stained protein ladder (Invitrogen), Lane 2: stock PA, Lane 3: stock PA with soil load, Lane 4: unexposed control, Lane 5: 1 minute exposure, Lane 6: 5 minute exposure, Lane 7: 10 minute exposure, Lane 8: 2 hours exposure.

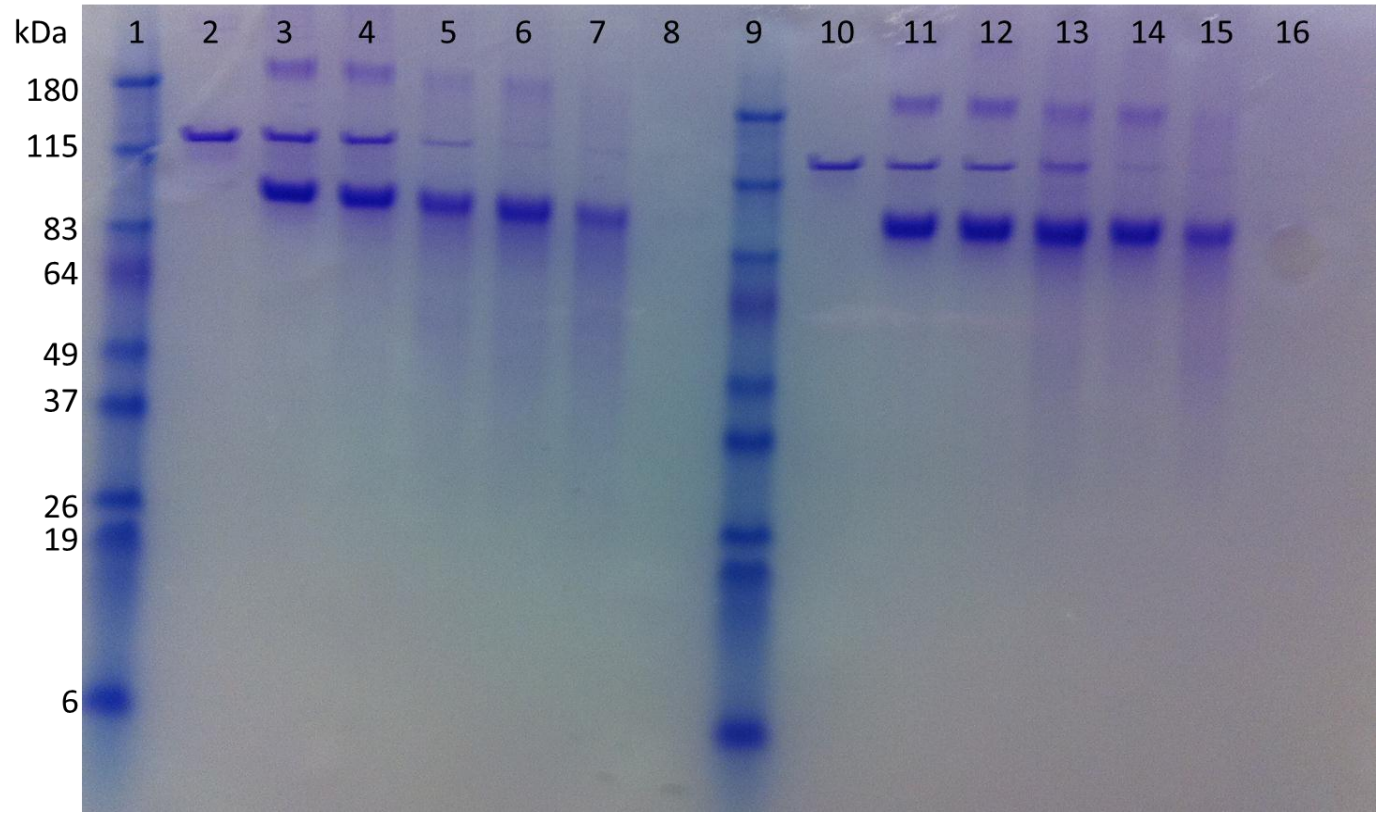



Figure 3.8.2: Protein gel electrophoresis of LF and PA exposed to VHP

Lane 1: Benchmark™ pre-stained protein ladder (Invitrogen), Lane 2: stock LF, Lane 3: stock LF with soil load, Lane 4: unexposed control, Lane 5: 1 minute exposure, Lane 6: 5 minute exposure, Lane 7: 10 minute exposure, Lane 8: 4 hours exposure, Lane 9: Benchmark™ pre-stained protein ladder (Invitrogen), Lane 10: stock PA, Lane 11: stock PA with soil load, Lane 12: unexposed control, Lane 13: 1 minute exposure, Lane 14: 5 minute exposure, Lane 15: 10 minute exposure, Lane 16: 4 hours exposure.

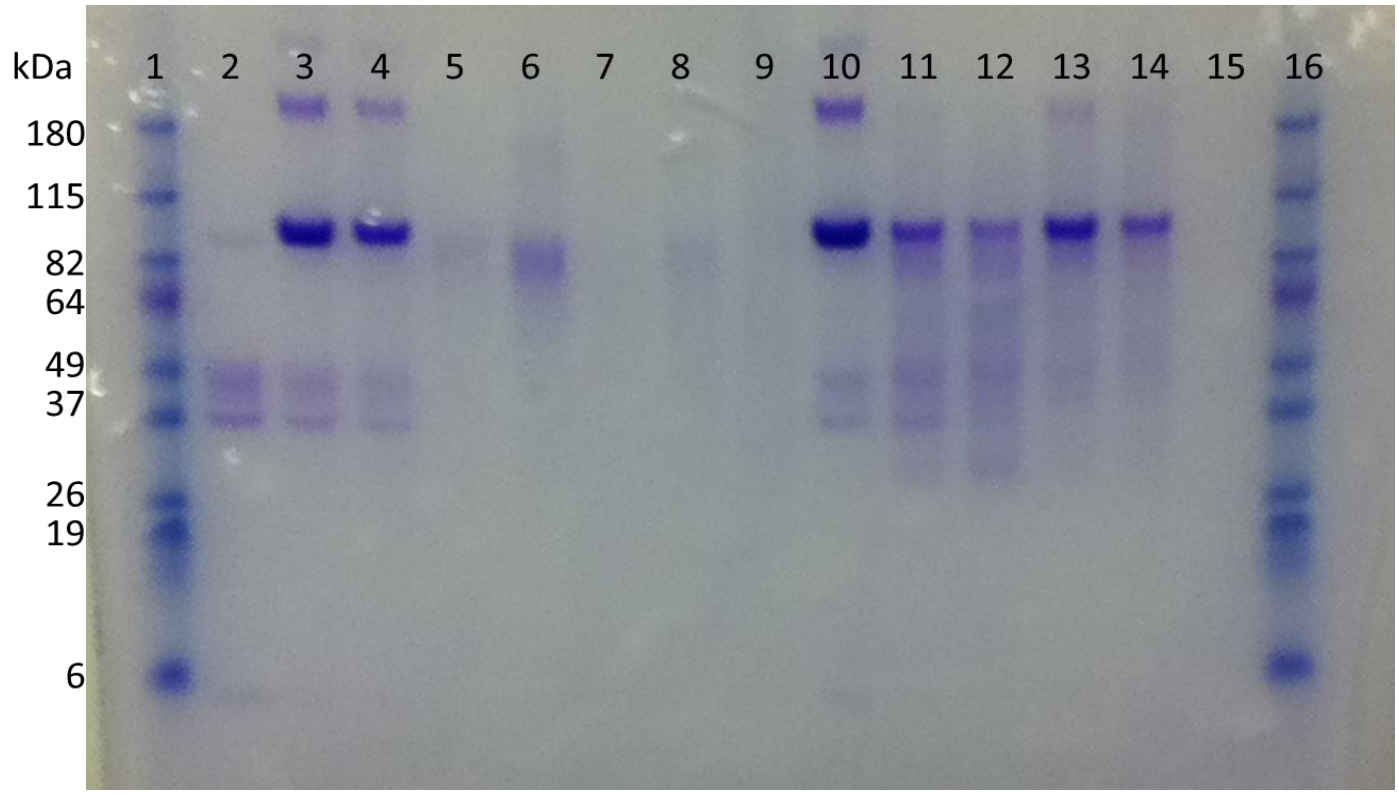



Figure 3.8.3: Protein gel electrophoresis of ricin exposed to GCD and VHP

Lane 1: Benchmark™ pre-stained protein ladder (Invitrogen), Lane 2: stock ricin, Lane 3: stock ricin with soil load, Lane 4: unexposed GCD control, Lane 5: 1 minute GCD exposure, Lane 6: 5 minute GCD exposure, Lane 7: 10 GCD minute exposure, Lane 8: 30 minute GCD exposure, Lane 9: 2 hour GCD exposure, Lane 10: unexposed VHP control, Lane 11: 1 minute VHP exposure, Lane 12: 5 minute VHP exposure, Lane 13: 10 minute VHP exposure, Lane 14: 30 minute VHP exposure, Lane 15: 4 hour VHP exposure, Lane 16: Benchmark™ pre-stained protein ladder (Invitrogen).

The stock ricin control in lane 2 (Figure 3.8.3) is seen as two bands at approximately 37-kDa and 49-kDa because the denaturing step cleaves the disulfide bond separating RTA from RTB. A very faint band is seen in lane 2 just over 82-kDa, which is most likely intact ricin that was not fully denatured by heating. Additional bands are seen in the control containing soil load (lane 3 Figure 3.8.3) and are also detectable in the unexposed controls. As seen in Figure 3.8.3, ricin was not detectable in samples exposed to GCD although soil load is still detectable in lower time points. Faint bands of RTA and RTB along with soil load are visible in ricin exposed to VHP for 1 and 5 minutes (Figure 3.8.3). With increased length of VHP exposure the RTB and RTA bands become fainter and more diffused indicating degradation, with no protein detectable in the sample exposed for 4 hours.

## 3.8 Ricin GFP Inhibition Assay

The CMV IE promoter was successfully amplified from the pQCXIP vector with the addition of *Bam*HI and *Xho*I restriction sites at the 3' and 5' ends. This PCR product was isolated and purified by gel extraction of a band of approximately 500 bp. Both the CMV IE promoter PCR product and pZsGreen1-DR vector were digested with *Bam*HI and *Xho*I creating sticky ends that were successfully ligated to create the CMV/pZGreen construct. The construct was transformed into chemically competent *E. coli* cells and a successful clone was chosen based on PCR screening, restriction enzyme digest patterns and DNA sequencing.

The isolated plasmid DNA of this clone was transfected in Vero 76 tissue culture cells and GFP expressing cells were observed microscopically. Repeat attempts were made at isolating a stable GFP expressing clone by serial dilution method. After isolation, a decrease in

GFP expression was observed in all clones. After several unsuccessful isolation attempts, the Ricin GFP Inhibition Assay was abandoned in favour of the MTT cytotoxicity assay.

# CHAPTER 4.0: DISCUSSION

Very little data have been published on the efficacy of detoxification methods against biological toxins. One of the major reasons for a lack of efficacy data on anthrax toxins is that if anthrax were to be used in a bioterrorism attack, the spore form, which does not produce toxins, would be the most likely mode of release due to its hardiness. In this case, the amount of toxin present would likely be very low and decontamination of the anthrax spores would be of greatest concern. It is because of this that most decontamination research is focused primarily on the *B. anthracis* spore. Despite this, determining the effectiveness of GCD and VHP against proteins such as the anthrax toxins does provide important data as it furthers the understanding of exactly how GCD and VHP kill complex microorganisms, which are composed of proteins among other molecules. Also these data are useful in determining effective decontamination methods for spaces in which large concentrations of anthrax toxins are manipulated or for the remediation of spaces accidentally contaminated. These data will also assist in determining decontamination conditions for other toxins that are more likely to be used in a bioterrorism attack, such as botulinum toxin, for which there are no efficacy data.

The ricin toxin is the form most likely to be used in a bioterrorism attack and in such an event the remediation of affected buildings would be of great concern. Although no published data are available on gaseous methods of inactivating ricin, a few studies have been conducted

on the efficacy of liquid disinfectants. In a 2008 study by Cole et al., sodium hypochlorite (bleach) and monochloramine were analyzed for their effectiveness against ricin using the MTT cell cytotoxicity assay and native fluorescence of amino acids for the detection of ricin degradation. Bleach was found to effectively destroy biological activity of the toxin, while monochloramine was less effective (Cole, Gaigalas et al. 2008). Bleach was also found effective at inactivating ricin using a neutral red cytotoxicity assay and protein gel electrophoresis (Mackinnon and Alderton 2000).

As toxins are not living organisms but proteins, testing the efficacy of decontamination is more complex than with bacteria and viruses, for which simpler methods of enumerating the number of organisms left after exposure are employed. Methods used for toxins must not only detect the presence of the toxin but also whether it has retained biological activity. Animal bioassays have often been used for analyzing the *in vivo* activities of toxins, but *in vitro* assays utilizing mammalian cell cultures can also be used. The MTT cytotoxicity assay is one of many methods for detecting cell death, which is an indirect way of measuring the enzymatic activity of the toxin.

The use of the MTT cytotoxicity assay in the detection of anthrax lethal toxin (LF combined with PA) has previously been investigated and optimized specifically as the toxin neutralization assay (TNA), for use in the evaluation of antibody responses to vaccines and immunotherapies (Hering, Thompson et al. 2004; Li, Soroka et al. 2008). In this application, the MTT cytotoxicity assay is used to measure the neutralization of lethal toxin by antibodies and results are reported as the ED50, which is the dilution of serum that neutralizes 50% of the cell cytotoxicity (Hering, Thompson et al. 2004; Li, Soroka et al. 2008). Although the rapid cell

death of macrophages caused by lethal toxin is irrelevant to human anthrax disease (Turk 2007; Moayeri and Leppla 2009) it is useful in the detection of lethal factor and protective antigen as both toxins must be present and fully active in order to reach the cellular target and cause death. This assay therefore not only detects the presence of the toxins but cell death also indicates that both toxins are fully active. This assay was found to be useful in testing the efficacy of VHP and GCD against anthrax LF and PA. Absorbance values detected and cell viability levels calculated for the unexposed controls and cells not treated with toxin were similar to those in these previous studies (Hering, Thompson et al. 2004; Verma, Wagner et al. 2008).

The MTT cytotoxicity assay was also able to detect the biological activity of ricin. The level of cell death observed for the unexposed ricin controls was similar to that seen in a previous study using the MTT assay for ricin detection (Halter, Almeida et al. 2009). Unexposed controls of ricin had higher levels of cell viability compared to similar controls of anthrax toxins, possibly indicating the ricin was less toxic to the cells at the concentrations used. Cells in contact with ricin for longer periods of time (up to 44 hours) have been noted to result in closer to 100% cell death (Cole, Gaigalas et al. 2008), but 22 hours contact time is generally chosen as it greatly shortens the length of the assay and produces consistent results (Cole, Gaigalas et al. 2008; Halter, Almeida et al. 2009). This higher minimum cell viability level was also observed to increase in later experiments. The toxicity of the unexposed ricin in VHP experiments was lower than that in the GCD experiments which were conducted at an earlier date.

One potential reason for the drop in ricin toxicity over time is the age of the tissue culture cells used for the cytotoxicity assay. Vero cells used for the many controls conducted for the assay and for GCD exposures were at passage 30-45 (since starting from liquid nitrogen storage)

and resulted in minimum cell viabilities of approximately 12-23% which increased with increasing cell passage. Minimum cell viabilities for the VHP exposures were the highest at closer to 30% and utilized cells of passage 50-60. This phenomenon did not occur in the anthrax testing, most likely because the macrophage cells were only used up to passage 25, after which a fresh stock of cells was started from liquid nitrogen storage. This protocol was used based on previous findings that cell passages greater than 25 greatly influenced the TNA in macrophage cells (Hering, Thompson et al. 2004; Li, Soroka et al. 2008). Higher passaged macrophage cells were found to be less susceptible to cell death caused by anthrax lethal toxin in the TNA (Hering, Thompson et al. 2004; Li, Soroka et al. 2008).

Changes in cell properties such as cell morphology, response to stimuli, growth rates, protein expression and cell signaling have been known to occur in cell lines at high passages (Briske-Anderson, Finley et al. 1997; Chang-Liu and Woloschak 1997; Sambuy, De Angelis et al. 2005; ATCC 2007) although the mechanisms behind these changes are unknown. As well these changes are cell line and application dependent and the passage level considered to be high differs between cell lines and applications (ATCC 2007). Vero cells have been used in different applications at passages higher than 60 without problems in the past and passages of 60 are generally not considered too high for Vero cells. Based on the data resulting in this study it appears Vero cells passaged higher than 45 should not be used for cytotoxicity assay detection of ricin in the future.

Both GCD and VHP were effective in reducing the cytotoxic effect of LF and PA on macrophage cells. In exposures of both methods PA was observed to cause more cell death than exposed LF potentially indicating PA to be more resistant to inactivation than LF. This result

could be misleading and not due to a difference in levels of inactivation between the two proteins, but instead caused by the different amounts of LF and PA needed to cause cytotoxicity. Seven molecules of PA must bind receptors and come in contact in order to form the PA heptamer needed to deliver a minimum of one and a maximum of three molecules of LF to the cytoplasmic target (Young and Collier 2007). Therefore similar levels of LF and PA degradation could result in different levels of cell death as more molecules of PA are needed to cause cell death. Unfortunately the cytotoxicity assay does not measure number of molecules, only the indirect outcome of the presence of these molecules.

VHP was found to be the most effective method for detoxifying both LF and PA even with presence of soil load. After only 5 minutes of exposure to VHP, cytotoxicity of both toxins was greatly reduced (Figure 3.3.3). Based on past research (Krishnan, Laframboise et al. 2006; Rastogi, Wallace et al. 2009) the presence of a soil load does not affect the efficacy of GCD, while it greatly affects VHP efficacy, therefore it is likely this is not the cause of the differences in efficacy. As GCD only reacts with highly reduced molecules (Luftman, Regits et al. 2008), while VHP is much less selective, GCD decontamination could result in less degradation of the toxins than VHP.

Ricin appeared to be more resistant than the anthrax toxins to both GCD and VHP, with GCD exposure resulting in the greatest reduction of cytotoxicity. Again, since the MTT cytotoxicity assay measures the level of cell death caused by toxin activity rather than the quantity of toxin molecules, these results may be misleading. It is known that ricin is more toxic than anthrax lethal toxin with a lethal dose of approximately 5-10 µg/kg (Bradberry, Dickers et al. 2003). Reports of the lethal dose for anthrax lethal toxin vary based on differing levels of

susceptibility between animals, with as low as 7 μg and as high as 250 μg causing death in rats (Moayeri and Leppla 2009). Therefore more lethal toxin is needed than ricin to achieve similar levels of cell death. This means that while the lower quantities of ricin remaining after GCD and VHP exposures are still cytotoxic, similar levels of anthrax toxins are not. This is most likely because only one molecule of ricin is required to reach the cytoplasm to cause cell death, as one molecule can inactivate 2000 ribosomes per minute (Olsnes 2004). Macrophage cell death caused by lethal toxin is much more complex and a full understanding of the cause has yet to be elucidated.

While VHP proved to be more effective than GCD against the anthrax toxins, the opposite was found for ricin. It is possible these results could be due to differences in the structure of the toxins and how GCD and VHP react with these molecules. Ricin B chain is quite different in structure from the anthrax toxins as it is a globular molecule lacking β sheets and α helices (Lord, Jolliffe et al. 2003). The toxicity of ricin is heavily dependent on RTB with similar type 1 RIPs being non-cytotoxic as they lack B chains (Lord, Jolliffe et al. 2003). It is possible that GCD is more effective at degrading globular structures such as RTB than VHP and differences in the degradation of the B chain would therefore greatly influence the efficacy of decontamination.

Another trend observed in the data collected from ricin decontaminations was a greater variability between replicates than in experiments involving the anthrax toxins. It is conceivable that the greater variability was caused by the lower quality of Vero cells used in these assays due to the higher passage number of the cells as previously mentioned. If this was the cause then one would expect to observe less variability in the GCD experiments as they were conducted using

lower passage cells. However this was not the case as similar levels of high variability were found in both GCD and VHP experiments (Figure 3.6.2). A more likely explanation for the increased variability is differences in the amount of active ricin able to reach its cytoplasmic target. The pathways utilized by ricin to reach the cytoplasm are complex and not fully understood, with only 5% of ricin that binds to surface receptors actually reaching its enzymatic target (Sandvig, Grimmer et al. 2000; Lord, Jolliffe et al. 2003; Olsnes 2004). At intermediate exposure time points samples will contain both active and degraded ricin, but with 95% of the ricin present not ever reaching the ribosome the ricin molecules retaining activity may not be detected, causing variability between replicates.

In general, gel electrophoresis confirmed results observed with the cytotoxicity assays and provided further insight into the detoxifying abilities of GCD and VHP. It was observed that in all gels, the molecular weight of proteins determined based on the protein ladder are slightly larger than expected based on protein sizes found in the literature. LF and PA are 90-kDa and 83-kDa respectively but are seen as approximately 115-kDa based on the protein standard used. Ricin is seen as two bands due to the denaturing step which breaks the disulfide bond between RTA and RTB. The proteins are estimated to be 40 and 50-kDa, while RTA is known to be approximately 32-kDa and RTB approximately 30- kDa. This is because the prestained ladder used is meant for estimating protein size and is not as accurate as an unstained standard would be (Invitrogen 2009). Although slightly inaccurate in determining protein size, the gels do detect the level of the degradation of the toxins caused by GCD and VHP.

GCD begins to degrade LF and PA right away as is seen by the more diffuse and smeared band in samples exposed for 1 minute and 5 minutes (Figure 3.8.1). Although no longer

detectable in further exposures, LF and PA are still cytotoxic as seen in Figure 3.3.3, most likely because the cytotoxicity assay is more sensitive than SDS-PAGE and Coomassie staining and is able to detect smaller concentrations of remaining toxin. The SDS-PAGE analysis confirms the cytotoxicity assay results indicating PA to be more resistant to GCD than LF as seen in Figure 3.8.1 where PA exposed to 1 minute of GCD is still visible as a distinct band while the LF sample contains a smear of bands. PA and LF, as well as the soil load proteins, are still visible as faint and slightly smeared bands in samples of up to 10 minutes of VHP exposure (Figure 3.8.2), although based on the cytotoxicity data VHP was better at inactivating the toxins. This highlights that protein gel electrophoresis methods alone are not enough to determine efficacy of detoxification. It is possible that the way in which VHP inactivates these proteins does not fully degrade the protein structure (as is the case with GCD), but may change the 3D structure making the toxin no longer functional.

Similar results were seen in SDS-PAGE analysis of ricin where GCD appears better at degrading the protein than VHP. Previous studies also found samples of ricin exposed to a different chlorine compound (sodium hypochlorite) had a similar banding pattern to the control when retaining cytotoxicity, while samples showing a reduction in cytotoxicity had negligible banding (Mackinnon and Alderton 2000; Cole, Gaigalas et al. 2008).

# CHAPTER 5.0: CONCLUSIONS

The MTT cytotoxicity assay was successfully utilized for the detection of anthrax lethal toxin and ricin in determining the efficacy of GCD and VHP decontamination. It was determined that further optimization of the assay would be useful in order to determine the limit

of Vero cell passage necessary to achieve maximum cell death in ricin controls. The GFP inhibition assay was not found useful in the detection of ricin due to difficulties in producing a stable GFP expressing cell line. Protein gel electrophoresis analysis provided additional insight into the efficacy of VHP and GCD against the anthrax toxins and ricin. Both decontamination methods were found to greatly reduce the activity of anthrax lethal factor and protective antigen, with the greatest reduction observed after four hours of VHP exposure. Ricin activity was also reduced by VHP and GCD exposure, but to a lesser extent due to the greater inherent toxicity of ricin. GCD exposure of two hours resulted in the greatest reduction in ricin activity. Differences in the efficacies of VHP and GCD have also provided information to assist us in further understanding the possible distinctions in the mechanism of protein inactivation of the two methods. The efficacy data has also indicated both GCD and VHP have great potential for use against biological toxins.

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## Appendix:

Table A.1: Primer sequences used for the generation of the pGFPds construct for the Ricin GFP protein inhibition assay

| Primer Name | Sequence 5'→3' | Added Restriction sites | Application used for |
|---|---|---|---|
| **CMVIEfwd** | GTACCTCGAGGTCCGTTACATAACTTACGGTAAATG | BamHI | Sense primer for CMV IE promoter PCR |
| **CMVIErev** | GTACGGATCCGATCTGACGGTTCACTAAACGAG | XhoI | Anit-sense primer for CMV IE promoter PCR and PCR colony screen |
| **pZG-MCSfwd** | TAGTTATTACTAGCGCTACCGGACTCAGATC | N/A | Sense primer for PCR colony screen |
| **Seq1fwd** | ATCAAGTGTATCATATGCCAAGTAC | N/A | pGFPds sequencing primer |
| **Seq2rev** | GAGTTGTTACGACATTTTGGAAAGT | N/A | pGFPds sequencing primer |
| **Seq3fwd** | CAGCAACGCGGCCTTTTTACGGTTC | N/A | pGFPds sequencing primer |
| **Seq4rev** | GGTTGCCGTACATGAAGGCGGCGGA | N/A | pGFPds sequencing primer |

Table A.2: Restriction digest reactions used in the generation of the pGFPds construct for the Ricin GFP protein inhibition assay

| **Reaction Reagents** | **CMV IE promoterPCR product** | **pZGreen-1 vector** | **Plasmid confirmation** |
|---|---|---|---|
| **NEB buffer 3 (10x)** | 5µl | 3 µl | 3 µl |
| **BSA (10mg/ml)** | 0.5 µl | 0.5 µl | 0.5 µl |
| ***Bam*HI (20,000 U/ml)** | 1.5 µl | 1 µl | 1 µl |
| ***Xho*I (20,000 U/ml)** | 1.5 µl | 1 µl | 1 µl |
| **DNA to be digested** | 41.5 µl | 2 µl | 1 µl |
| **Sterile water** | 0 µl | 22.5 µl | 23.5 µl |