DISTRIBUTION AND DETECTION OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN AFTER INTRAPERITONEAL AND INTRAGASTRIC ADMINISTRATION USING THE MURINE MODEL

By

ANDREAS MARKUS KELLER

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

This dissertation is dedicated to my parents. Karl-Heinz and Charlotte; to my wife Cecilia; to my daughter Dominik; to my Onkel Peter; to my Omi Lotti; and with remembrance to my late sister Sylvia; and Opi Paul and Omi Elisabeth, for their never ending love, patience, enthusiasm, dedication, understanding and support.

Thank you, God, for such a wonderful family.

ACKNOWLEDGMENTS

I would like to thank Dr. James A. Lindsay, my major advisor (I prefer the German word "Dr. Vater," since advisor is a too simplistic a term to describe a mentor, role model and benefactor), from the bottom of my heart, for his never-ending support, patience, persistence, guidance, motivation, commitment, encouragement, enthusiasm, and enlightenment in every step of my doctoral research, graduate program, and personal life. I am extremely grateful to the other members of my doctoral committee, Dr. Douglas L. Archer, Dr. Sean F. O'Keefe, Dr. Mark L. Tamplin, and Dr. Ramon D. Littell, for their interest, advice, suggestions, review of manuscript, and supportive role in my research. I am deeply appreciative of the Food Science and Human Nutrition Department, USDA, and NIH for providing me with graduate assistantships and all other funding. I would like to thank F. Morgan Wallace for his advice, support, and friendship. I also give my sincerest thanks to Annette S. Mach for conducting the tissue culture studies, and above all her suggestions, assistance and guidance in the lab, making it a fun, efficient, sound, and safe environment to work in. Finally, my sincerest thanks go to my friend Dr. Antonio A. Figueiredo.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

DISTRIBUTION AND DETECTION OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN AFTER INTRAPERITONEAL AND INTRAGASTRIC ADMINISTRATION USING THE MURINE MODEL

By

ANDREAS MARKUS KELLER

May, 1997

Chairman: Dr. James A. Lindsay
Major Department: Food Science and Human Nutrition

Clostridium perfringens has been described as the most important anaerobic pathogen of man, and is considered the most common cause of enteric diseases in animals. Virulency of the bacterium is related to the production of at least 15 different protein-toxins, many of which are lethal. Diseases associated with *C. perfringens* infections and production of these protein-toxins include myonecrosis (gas gangrene), necrotic enteritis, antibiotic associated diarrhea, sudden infant death syndrome and food poisoning in man and animals.

To understand these changes, the whole body distribution of CPE after either intraperitoneal and intragastric administration was determined, using the murine model. Results showed that CPE appeared to have three different modes of distribution and activity which were time and concentration dependent. Nonlethal levels induced enterotoxigenic symptoms, while sublethal levels induced symptoms described as parasympathomimetic. Administration of lethal levels induced two patterns of death, first, a sudden death induced within minutes of CPE administration, and second, a nonabrupt death that required several hours for manifestation. Animals expressed symptoms of respiratory distress, shock and multiple organ failure, similar to the action of a superantigen.

From the murine model studies, the following parallels may be suggested for CPE toxicosis in humans. Nonlethal levels of CPE causes a toxicosis similar to a self-limiting foodborne illness. Systemic absorption of sublethal levels of CPE induce a neurotoxicosis, from which healthy individuals would likely recover. However, death could occur in immunocompromised persons or the elderly. Systemic absorption of lethal levels of CPE will cause death in both healthy and immunocompromised individuals. The finding of a "sudden" pattern of death after lethal ingestion is pivotal, since this mimics the suggested response of some at-risk infants to CPE toxicosis, and supports the role of CPE as a trigger in some cases of the sudden infant death syndrome.

CHAPTER 1
INTRODUCTION

Clostridium perfringens foodborne illness (FBI) is associated with enterotoxin(s) from type A strains and is the third most common cause of bacterial FBI in the USA following *Salmonella* spp. and *Staphylococcus aureus*. Annual costs of FBI associated with *C. perfringens* in the USA and Canada are estimated to be higher than $200 million. Mortality rates from *C. perfringens* FBI are dependent on age and immune status, and the debilitated, immunocompromised, young and elderly are at high risk. In the USA mortality rates may be as high as 4% (Janssen et al., 1996). Besides causing foodborne illness *C. perfringens* has been associated with some unusual disease states, for example wound infections and sudden infant death syndrome (SIDS) (Lindsay et al., 1993, 1994; CDC, 1994; Lindsay, 1996). Studies indicate that *C. perfringens* infections and type A enterotoxin appear to be associated with 50-80% of the approximately 7,000 SIDS deaths/year in the USA (Lindsay, 1996.

Consequently, there has been a dramatic increase of interest in the bacterium's pathogenicity, virulence determinants, and the signals controlling expression of these determinants. Studies show that *C. perfringens* type A enterotoxin (CPE), which is produced in the highest amounts during bacterial sporulation, may have the ability to

modulate the host defense system, by acting as a superantigen and exerting immunomodulatory effects on various lymphoid cell populations, thus playing an important role in the overall pathogenesis of the organism (Lindsay, 1996). Superantigens may activate and stimulate up to 1 in 5 T-cells as compared to a classical antigen which normally stimulates 1 in 10,000 T-cells. Stimulation of T-cells may lead to the induction of cytokines, such as interferon-γ, tumor necrosis factor-ß, interleukins, and others, usually in a cascade. Cytokines may cause a decrease in blood pressure, shock, respiratory distress, multiple organ failure and death (Lindsay, 1996). Most T-cells activated by superantigens are useless in fighting infections, and even worse they could unleash an autoimmune attack, driving the immune system into a self destructive frenzy, hurting the individual instead of protecting. Superantigens also have the ability to trigger the cell death of cells they excite, thus weakening the body's defense system (Johnson et al., 1992).

Studies on superantigens have predominantly focussed on the effects of staphylococcal enterotoxins in animals as a model of human toxicosis (Cerami, 1992; Tracey and Cerami, 1993; Fleischer, 1994). Still numerous important questions such as: the functional role of bacterial superantigens, and how superantigens with different structures can interact with major histocompatibility complex (MHC) and T-cell receptors remain unanswered.

Recent data indicates that CPE has superantigenic properties; however, it has not been determined whether the toxin's enterotoxigenic, cytotoxigenic and

parasympathomimetic properties are linked to superantigenicity (Bowness et al., 1992; Lindsay, 1996). Currently there are several areas under study relative to CPE: i. the mechanics of pathogenicity; ii. the structure of the CPE genes and the signals controlling regulation and expression; iii. the mechanics of CPE action at the cellular level and interactions with host molecules; iv. and the mechanisms/role of superantigenicity. Various laboratories are currently working on: i. cloning the enterotoxin genes to determine structure and function, and mechanisms for regulation; ii. CPE receptor binding and gross mechanisms of action; iii. and CPE's mechanisms of superantigenicity.

The overall aim of this dissertation was to investigate the pathophysiological responses after intraperitoneal and intragastric administration of CPE using the murine model. This evaluation may explain how CPE becomes distributed during toxicosis, which organs were specifically affected, and describe the numerous sequela and pathophysiological changes that may lead to death. Within the overall aim was the development of a murine model that would explain the mode of CPE distribution after intragastric administration, thus being able to draw a comparison with the SIDS model proposed by Lindsay et al. (1994), and Lindsay (1996), and possibly determining the events involved and leading to infant death.

The specific objectives to this study were as follows:

1. To identify the murine organs and tissues that bind CPE after *in vivo* administration of CPE.
2. To determine and compare the number of CPE cell receptors in each organ.
3. After intraperitoneal CPE administration, to investigate the whole-body distribution of CPE.
4. Describe the animals' symptoms during toxicosis after intraperitoneal and intragastric CPE administration, physical and pathophysiological changes, and findings at necropsy.
5. After intragastric CPE administration investigate the whole-body distribution of CPE .
6. Propose a murine model for the whole-body distribution of CPE after intragastric administration.

The results will hopefully elucidate the CPE distribution mode, delineating the affected organs and pathophysiological changes possibly responsible for the illness and death. Ultimately the murine model will provide a platform for analogy in the development of human FBI and SIDS.

CHAPTER 2
REVIEW OF THE LITERATURE

Clostridium perfringens

Clostridium perfringens (*Clostridium welchii*) has been described as the most important anaerobic pathogen of man (Lindsay, 1996) and is considered the most common cause of enteric diseases in animals (Hobbs et al., 1953; Bartoszcze et al.,1990; Songer, 1996). Virulence of the bacterium is related to the production of at least 15 different protein toxins, many of which are lethal. Diseases associated with *C. perfringens* infections and production of these protein toxins include myonecrosis (gas gangrene), necrotic enteritis, antibiotic associated diarrhea, sudden infant death syndrome and food poisoning in man (MacLennan, 1962; Smith, 1979; Fekety et al., 1980; McDonel, 1980; Rood and Cole, 1991; Lindsay, 1996) and lamb dysentery, ovine, bovine and equine enterotoxemia, and pulpy kidney disease of sheep and other animals (McDonel, 1980; Niilo, 1980; Sterne, 1981; Songer, 1996).

Classification

C. perfringens strains are initially classified into a series of different types (A-E) based upon their production of one or more of the major toxins, alpha, beta, epsilon and iota. Toxin production is verified by neutralization with type specific antisera using mice; however, the process is very tedious, expensive and relies upon

the unnecessary use of animals. PCR-typing techniques are becoming more available and have proven to be reliable under some circumstances. However, considerable discussion has ensued as to whether using a classification method based on four toxins is correct since it appears that not all strains contain the alpha-toxin (phospholipase C) a current defining characteristic of *C. perfringens* (Lindsay, 1996).

C. perfringens is the most widely distributed pathogenic bacterium. The organism is a Gram-positive, rod-shaped, variably sized (0.6-2.4 x 1.3-1.9 μm) encapsulated, nonmotile, spore former, occurring singly or in pairs. Vegetative cells are mostly square-ended rods but some strains have rounded ends. The bacterium usually grows very quickly and can have a generation time of 7 minutes in an optimal meat-containing environment (Labbe, 1989). Although *C. perfringens* can be isolated relatively easily, colony appearance on solid medium varies with organism type. Isolation requires the differential use of various antibiotics, the presence or absence of iron and sulfite, and incubation temperature. *C. perfringens* are resistant to many antibiotics which inhibit other anaerobes or facultative anaerobes. Sulfites are reduced to sulfides which in turn react with iron, forming a precipitate that renders *C. perfringens* colonies black. Selective media commonly used for isolation and enumeration are sulfite polymyxin sulfadiazine (SPS) agar; tryptone sulfite neomycin (TSN) agar; Shahidi Ferguson perfringens (SFP) agar; D-cycloserine blood agar; oleandomycin polymyxin sulfadiazine perfringens (OPSP) agar; tryptose sulfite cycloserine (TSC) agar; and egg yolk free tryptose sulfite cycloserine (EY-free TSC)

agar. Although colony growth is good at 37°C, incubation at 46°C especially on TSN is highly selective. If the organism is present in foods or feces in the spore state, samples are usually heat shocked at 75°C for 15 minutes before plating. Lindsay (1996) suggested that since spore heat resistance is correlated with CPE synthesis, that spores should be heat shocked at both low (75°C) and high (100°C) temperatures to ensure complete activation of all sub-populations within a sample.

C. perfringens is not a strict anaerobe since growth occurs between +125 and -125 millivolts (Eh). Thus the organism is described as aerotolerant. Vegetative cells are sensitive to high Eh during lag- and early log-phase, but oxygen extends the lag phase and growth can be stimulated by lowering the Eh. Oxygen peroxides, however, reduce colony growth, and cell/spore counts. The optimum growth temperature varies from 43-47°C based on organism type, and the T_{min} is usually 20°C and the T_{max} 50°C. Thus the vegetative cells are heat tolerant. Some strains are known to grow slowly at 15°C but these are the exception. Refrigeration at < 5°C and freezing can decrease the number of vegetative cells and spores. Vegetative cells are very sensitive to acid environments during log phase, but during stationary phase cells are resistant. The optimum pH range for growth and toxin production differ. Optimum growth occurs at pH 6.0-7.5, while pH under 5 or above 8.3 are extremely inhibitory. The optimum pH for toxin production is 7.0 for alpha toxin, 7.5 for beta, and 7.2 for epsilon and theta (Hobbs, 1979; Labbe, 1989). Optimal toxin production occurs between 30-46°C. The water activity (a_w) range for growth is 0.93-0.97

depending on the solute controlling the a_w of the substrate. During sporulation, the a_w is a more significant growth limiting factor than for vegetative growth (Hobbs, 1979; Labbe, 1989). Many strains tolerate curing agents and smoking when a suitable growth temperature and pH are maintained. Complete growth inhibition occurs at 8% NaCl, 1g/kg $NaNo_3$, 400mg/kg $NaNO_2$, and with a combination of 5.3% NaCl with 25mg/kg nitrite (Hobbs, 1979; Labbe, 1989).

In nature, *C. perfringens* is usually found as a spore which becomes metabolically active only when it encounters a suitable substrate. Thus the bacterium is regarded as a r-strategist (Lindsay, 1996). Spores produced in sporulation media are subterminal and oval in shape. Sporulation is strain, temperature and medium dependent. A range of 32-40°C is appropriate for most strains (Lindsay, 1996) and maximum spore production is reached in 6-8 hrs. Many *C. perfringens* strains have different nutrient requirements, thus the choice of sporulation medium is critical. Duncan and Strong (DS) medium (Duncan and Strong, 1968) with some minor adjustments is used by most laboratories. The addition of either starch, raffinose, amylopectin, amylose, glucose, maltose and methylxanthines to DS is known to increase spore production. however, complete sporulation is never observed (Labbe, 1989; Lindsay, 1996). Five to ten percent sporulation is considered usual, and 50% exceptional. Some strains are known to have almost no spore formation ($< 0.001\%$) even under the optimal conditions. *C. perfringens* spores are relatively heat resistant D_{100} 15 min, however, strain variation is known. Non-hemolytic strains have a

decimal reduction time (D_{100}°C) of 6-17 min, whereas hemolytic strains have a D_{100}°C of 0.1-0.5 min. Heat activation is also strain dependent. Some strains only require 60°C for 5 min, although 10-20 min at 75-80°C is usual (Labbe, 1989). Some type A strains are highly heterogeneous with a sub-population activated at 75°C, and another at 100°C (Lindsay, 1996). Spore radiation resistance varies from 1.2 to 3.4 kGy, and radiation resistance parallels heat resistance (Labbe, 1989). *C. perfringens* spores are also highly resistant to curing agent at concentrations of 21.5% NaCl, 1.8 g/l $NaNO_3$ and 1.2 g/l NaO_2 (Labbe, 1989). Phenolic antioxidants are inhibitory or bacteriocidal depending on the compound and concentration used (Labbe, 1989).

C. perfringens Foodborne Illness

History

Although an outbreak of *C. perfringens* foodborne illness (FBI) was first described by Klein in 1895, it was not until 1943 when Knox and McDonald in the United Kingdom and McClung (1945) in the United States made the association between the organism and the FBI. Persons afflicted with the illness expressed symptoms of severe abdominal pain, mild chronic to explosive diarrhea accompanied by nausea. From 1947-9 there were many reported outbreaks of FBI in Germany with a large number of fatalities. Unlike the illnesses described in the UK and USA a few years earlier, the German patients suffered from severe gross hemorrhagic enteritis (enteritis necroticans: Darmbrand) where the bowel was completely

desquamated. Thus it was obvious that *C. perfringens* was responsible for two completely different types of FBI. The dilemma was finally resolved by Hobbs et al. in 1953 who unequivocally showed that type A strains were responsible for the mild form of FBI, and type C strains for the more dramatic-lethal form. Subsequent studies by Duncan and Strong (1969), Hobbs (1979), Niilo (1975), and Tsai and Riemann (1975a; 1975b) showed that the type A FBI was caused by an enterotoxin, now termed CPE, produced during cell sporulation. In 1967 a milder form of necrotic enteritis was observed in New Guinea. This type of outbreak was subsequently found to be common, and coincided with traditional pig feasting. A heat sensitive strain of *C. perfringens* type C was found to be the causative agent of the disease, now referred to as pigbel (Murrell and Walker, 1991). Studies during the last 20 years have shown that Darmbrand and pigbel result from the consumption of type C vegetative cells which proliferate in the gut producing beta-toxin. The diet of persons afflicted with the diseases is usually lacking in proteases needed for enzymatic digestion of the beta-toxin, or as in New Guinea, protease inhibitors are present in the bowel due to the consumption of sweet potatoes, the staple diet of the natives. Darmbrand and pigbel are now relatively rare due to immunization of the susceptible populations with beta-toxoid. However, in some inhospitable regions of New Guinea where cannibalism still occurs the disease is common and death usually occurs after infection (Murrell, 1989).

Type A Foodborne Illness: General Characteristics

While type C necrotic enteritis is rare, type A FBI is very common and these strains are associated with about 10% (third most common in the USA) of all bacterial FBI (Bean and Griffin, 1990). An infection may be caused by the ingestion of foods contaminated with $>10^6$-10^7 vegetative cells/gram (Hobbs, 1979). This contamination usually results from temperature abuse of a prepared food. Vegetative cells that survive the stomach acids, enter the small intestine, multiply and sporulate. During sporulation some cells synthesize CPE, and upon cell lysis the enterotoxin is released in the intestinal lumen where it attaches to villous enterocytes to act cytotoxically and histopathologically, or is also absorbed systemically where it may additionally be parasympathomimetic, cardiotrophic or superantigenic (Lindsay, 1996). Illness usually develops 8-24 hours after ingestion, and is resolved 12-24 hours after onset (McDonel, 1980; Labbe, 1989). The classical symptoms are severe abdominal cramps with mild to explosive diarrhea, accompanied by nausea. Vomiting is rare, although fever may occur. Death rates are usually low in immunocompetent individuals, however, in the young, elderly or immunocompromised persons the mortality rate is 3-4% (Janssen et al., 1996).

Type A FBI Outbreaks

From 1973-87 the Center for Disease Control (CDC) reported 1994 outbreaks of *C. perfringens* type A FBI in the USA. These outbreaks involved 12,234 cases and 12 deaths. The actual number of FBI cases/year is estimated to be around 650,000 with 7-10 fatalities/year (Bean and Griffin, 1990). *C. perfringens* outbreaks are often large, with a mean of 25 cases/outbreak (Todd, 1989; Bean and Griffin, 1990; CDC, 1994). There have been several serious outbreaks during the last two years traced to St. Patrick's Day meals, the first, in Cleveland, Ohio, involving 156 persons. Corned beef boiled for three hours was cooled slowly at room temperature before refrigeration. Four days later the meat was reheated at 48.8°C and consumed in sandwiches several hours later after preparation. The second occurred in Virginia where 86 people became ill. Commercially prepared, frozen-brined corned beef was cooked in large pieces, refrigerated and reheated with a heat lamp for 90 minutes before consumption (CDC, 1994). The third outbreak in a British hospital, involved 17 patients. Cooked vacuum-packed pork was cooled slowly at a commercial meat preparation facility, transported to the hospital, slowly reheated then consumed.

Temperature abuse during cooking (inadequate cooking) and improper cooling or holding temperatures account for 97% of type A FBI outbreaks. Since the bacterium is heat tolerant and its spores have an high resistance, incomplete cooking of contaminated foods may not kill all vegetative cells and will likely promote germination of spores and rapid outgrowth. Other factors often associated with type

A FBI include contaminated equipment, and improper personal hygiene. Prevention and control of type A FBI can be accomplished by thoroughly cooking foods and ensuring that high internal temperatures necessary to destroy the bacterium's spores are achieved. Cooked foods should be cooled quickly and either stored at refrigeration temperatures or consumed immediately.

Identification of *C. perfringens* FBI Outbreaks

To successfully identify *C. perfringens* type A FBI outbreaks several bacteriological criteria have to be fulfilled. Public health agencies may identify an outbreak by 1) finding the contaminated food and determining that it contains $>10^5$ *C. perfringens* vegetative cells/gram, 2) finding that patients have $>10^9$ *C. perfringens* spores/gram feces, 3) finding that patients express the same serotype of *C. perfringens* or, the same serotype should be found in both contaminated food and in patient feces (Stringer, 1985; McClane, 1992). A problem with these criteria is that many elderly individuals naturally have high *C. perfringens* spore counts in their stool. Further, many isolates from the U.S. and Japan are unusual and cannot be serotyped (Saito, 1990; McClane, 1992). Thus, it is now recommended that the diagnostic criteria for *C. perfringens* type A food poisoning outbreaks should include 1) the presence of CPE in feces FBI patients, and 2) isolation of *C. perfringens* type A strains carrying the *cpe* gene from feces or foods associated with the FBI. Detection of CPE in feces is a partial indicator of *C. perfringens* type A FBI and

several serologic assays (ELISA/reverse-passive latex agglutination:RPLA) are commercially available. A problem with these assays is that fecal samples have to be collected and examined immediately (Bartholomew et al., 1985; Stringer, 1985; Birkhead et al., 1988; Labbe, 1989; McClane, 1992). There is also the problem that a contaminating molecule produced by all *C. perfringens* strains (CPE positive and negative strains) cross reacts with antisera to CPE. Thus CPE detection should be corroborated with *cpe* gene probe assays, which are relatively easy to perform (Labbe, 1989; Kokai-Kun and McClane, 1996; Lindsay, 1996). However, there is an additional concern. In a recent survey of *C. perfringens* associated FBI cases where all the microbiological and serological criteria were met, *cpe* gene probe analysis revealed only 59% of associated strains were *cpe*-positive. Lindsay (1996) recently argued that results using even the latest molecular biology identification methods for *C. perfringens* should be treated with caution.

The reasons for this caution are as follows. All enterotoxigenic *C. perfringens* type A carry the *cpe* gene, however, CPE positive isolates account for $< 5\%$ of all *C. perfringens* isolates found globally. Thus strains capable of causing FBI present a very small sub-group within the ubiquitous and regularly encountered *C. perfringens* (Van Damme-Jongsten et al., 1989; Saito, 1990; Kokai-Kun et al., 1994). CPE-A positive strains have been isolated from feces collected from 6% of healthy food handlers, suggesting that humans may serve as reservoirs for *C. perfringens* type A strains (Hobbs, 1979; Saito, 1990). The issue of whether animals are indeed potential

reservoirs of enterotoxigenic *C. perfringens* remains questionable. In human FBI causing strains *cpe* is chromosomally encoded, whereas in veterinary isolates *cpe* is plasmid-borne (Cornillot et al., 1995). Only one plasmid encoded strain has been isolated from human *C. perfringens* FBI cases (Katayama et al., 1996).

C. perfringens and Pathogenicity

Virulence Factors Contributing to *C. perfringens* FBI

The association between cpe and *C. perfringens* FBI, although not formally proven by Koch's molecular hypothesis, is accepted by researchers. This acceptance is based on strong evidence from a number of sources, such as epidemiological studies where patients have detectable enterotoxin levels in their stools (80-100% of patients fecal samples tested CPE positive) (Bartholomew et al., 1985; Birkhead et al., 1988). CPE ingestion is known to cause illness with the same symptoms in experimental animals (Hobbs et al., 1953; McDonel and Duncan, 1975; Bartholomew et al., 1985; Birkhead et al., 1988). When either purified CPE or *C. perfringens* type A strains were fed to human volunteers they developed FBI symptoms, but patients fed CPE negative strains showed no illness (Skjelkvale and Uemura, 1977). It has also been shown that the effects of CPE in animal models can be neutralized with CPE specific antiserum (Hauschild et al., 1971).

Molecular Biology of CPE

The complete *cpe* gene has been cloned and sequenced allowing studies on the regulation and expression of the gene with regards to sporulation, and for the construction of various nucleic acid probes (Van Damme-Jongsten et al., 1989; Czeczulin et al., 1993; Cornillot et al., 1995; Lindsay, 1996). In type-A food poisoning isolates the *cpe* gene is present as a single chromosomal copy in the hypervariable C region of the chromosome. This hypervariable region is thought to contain mobile genetic elements (transposon or lysogenized phage) that allow the transfer of *cpe* to other *C. perfringens* strains (Canard et al., 1992). The plasmid encoded veterinary isolates are thought to represent an example of this type of mobility. The *cpe* gene can also be transferred and expressed in non-enterotoxigenic type A, B and C strains. A comparison of open reading frame (ORF) sequence data strongly suggests that CPE produced from different type A strains, or different variants of the same strain are similar, but not identical (Czeczulin et al., 1993, 1996). Although microsequence variation occurs, the *cpe*-ORF generally appears to be highly conserved. Enterotoxigenic veterinary and food poisoning strains have slightly different regulatory factors preceding the *cpe* ORF (Brynestad et al., 1994). A 45-base pair insertion about 265 nucleotides upstream from the start of the *cpe* gene has been detected in three strains, implying at least two types of *cpe*-promoter regions in foodborne isolates (Melville et al., 1994). The insertion does not appear to alter the starting point of *cpe* transcription in *C. perfringens*, although transcription was

significantly altered when the *cpe* gene was cloned into *B. subtilis*. The reason for this promoter diversity remains undetermined, although geographical segregation appears not to be a factor. The *cpe* gene may be associated with at least two repetitive sequences, one of which is a known insertion sequence (IS*1151*) (Daube et al., 1993; Brynestad et al., 1994; Cornillot et al., 1995). In human FBI strains, when *cpe* was chromosomally located, several factors were observed: first, the gene was preceded by a repeated sequence, the *Hind*III repeat and ORF3, which is homologous to a gene present on an IS element, and second, the gene was always present on a 5 kb *Nru*I fragment. In contrast, strains isolated from domestic livestock where *cpe* was plasmid encoded, the gene was close to IS*1151*, not linked to the *Hind*III repeat, generally preceded by ORF3, but never encoded on a *Nru*-fragment (Cornillot et al., 1995).

Sporulation and CPE

The molecular basis for regulation of CPE expression is not completely understood. Several key factors are now accepted: a) CPE expression is sporulation associated, b) CPE is not a structural component of the spore coat and, c) CPE is not a post-translationally processed product of a 52 kDa precursor molecule. The association between sporulation and CPE synthesis was first made by Duncan et al. (1972) who showed that CPE synthesis could be blocked by early stage sporulation mutants *spoO* but not but not by later stage *spoV* mutants. Subsequent work

confirmed these observations, indicating that although some CPE was synthesized during vegetative growth due to leaky gene regulation, CPE synthesis was indeed sporulation controlled, possibly by a global regulator such as a sigma factor (Duncan et al., 1972; McDonel, 1986; Czeczulin et al., 1993; Kokai-Kun et al., 1994; Lindsay, 1996). CPE expression is an exclusive trait of sporulating cells since although the *cpe* gene can be transferred to *E. coli*, transformed cells do not express the toxin. Enterotoxin production starts soon after sporulation is committed. Synthesis peaks 6-8 hrs into sporulation (Smith and McDonel, 1980; McDonel, 1986) and CPE comprises up to 10-20% of the total cellular protein content (Czeczulin et al., 1993).

The *cpe* mRNA may be transcribed as a monocistronic message, and its transcription starts approximately 200 base pairs (bp) upstream of the CPE translation start site. This promoter region does not show any significant sequence homology with other known bacterial promoters (Melville et al., 1994; Czeczulin et al., 1996). The *cpe* mRNA has an exceptionally long half-life of 58 minutes in sporulating cells, and this could in part be the reason for high CPE expression (Labbe and Duncan, 1977). Increased mRNA stability may result from a stem-loop structure localized 36 bp downstream of the 3' end of the *cpe* ORF. This region may also function as a rho-independent transcriptional terminator to regulate expression (Czeczulin et al., 1993). A similar situation occurs in the *seb* gene, where a palindromic sequence occurs 40 nucleotides downstream from the TGA stop codon. The hairpin-structure

is followed by an thymine-rich region that may function as a rho-independent transcriptional terminator (Gaskill and Khan, 1986; Jones and Khan, 1986).

CPE does not fit the classical definition of an exotoxin because the molecule does not contain a leader sequence, nor is it transported through the cell membrane. CPE production takes place in the mother cell cytoplasm, where it accumulates. When CPE synthesis is excessive, paracrystalline inclusion bodies are often formed or toxin is trapped between spore coat layers. Toxin is only released as a consequence of mother cell lysis at stage VII of sporulation (McDonel, 1980; Labbe, 1989; Czeczulin et al., 1993; Lindsay, 1996).

Biochemistry of CPE

CPE is a single polypeptide composed of 319 amino acids. The molecule has an isoelectric point of 4.3 and a M_r of 35,317 Daltons (McClane, 1992). The protein is heat labile and is inactivated by heating for 5 min at 60°C (McDonel, 1986). The toxin is denatured by pH extremes, but some proteolytic enzymes do not affect its stability (McDonel, 1986). Indeed trypsinization activates CPE threefold *in vitro* by cleavage of the first 23 amino acid residues respectively from the N-terminus. Chymotryptic removal of the first 36 amino acid residues from the N-terminus increases cytotoxicity twofold. These data strongly support the hypothesis that proteolytic digestion of the toxin in the intestinal lumen activates CPE during FBI (Granum and Richardson, 1991; McClane, 1992).

There are some data indicating that at least two different CPE molecules are synthesized, the well-characterized CPE also known as CPE-A and the less common CPE-86. The CPE-86 toxin is derived from a *C. perfringens* coatless spore mutant. Some data indicated a high degree of nucleic acid sequence and N-terminal protein homology between CPE-A and CPE-86 (Wojciechowski, 1995). However, other data from amino acid analysis and matrix-assisted laser desorption (MALD) ionization mass spectroscopy comparisons suggested distinct structural differences (Lindsay et al., 1985; Wojciechowski, 1995; Lindsay, 1996). These conflicting results led Wojciechowski (1995) to suggest that although the nucleic acid sequences were the same, any differences in protein structure either resulted from post-translational modification, or conformational differences as a result of post-translational modification. This hypothesis remains to be proven (Lindsay, 1996). CPE-A and CPE-86 appear to have similar mechanisms of action with regard to *in vitro* cell proliferation (McClane and McDonel, 1979; Lindsay, 1988) and cytokine modulation in several different human and animal cell lines (Mach and Lindsay, 1994, 1997). Although CPE-86 is biologically more active *in vivo* and *in vitro* (McClane and McDonel, 1979; Lindsay et al., 1985; Lindsay, 1988), both toxins are equally mitogenic in the J774Mϕ cell line (Mach and Lindsay, 1997).

The structure-function relationship for CPE is complicated in part by the inconsistencies in structural data. Examination of CPE-A secondary structure by UV circular dichroism predicted totally different values to those derived from the amino

acid composition (Granum and Harbitz, 1985; Lindsay et al., 1985). Additionally, attempts to predict CPE-A secondary structure using 9 different structural models showed conflicting putative conformations that were consistent in only two areas (Granum and Stewart, 1992). Possibly the high percentage of hydrophobic amino acids in both protein-toxins causes equivocal results by different methods. The 3D structure of CPE-A examined by differential spectroscopy, and by titration of accessible amino groups suggests a 2-domain structure, where the N-terminal portion of the protein is cytotoxic and the C-terminal portion is the binding portion (Granum and Whitaker, 1980; Whitaker and Granum, 1980; Granum and Stewart, 1992). This concept is strongly supported by MAb and PAGE studies (Lindsay et al., 1985; Hanna et al., 1992). X-ray analysis of either protein-toxin could possibly provide more definitive information. Unfortunately, although crystals of CPE-A have been produced, they are too small in both quantity and size for analysis (Granum and Stewart, 1992).

Structure-Function and Vaccines

Through the use of recombinant peptide fragments, and the generation of monoclonal antibodies, the regions of activity within the CPE protein have been partly mapped (Horiguchi et al., 1987; Hanna et al., 1989, 1991, 1992; Granum and Richardson, 1991; Hanna and McClane, 1991). Although CPE is a single unit polypeptide, similar to most bacterial toxins, CPE presents two distinct regions, a

hydrophobic toxic fragment and a hydrophilic binding fragment. As previously noted proteolytic digestion of the N-terminus with either trypsin or chymotrypsin activates CPE, with a concomitant increase in CPE cytotoxicity (Granum et al., 1980). Amino acids 37 through 171 in the first half of the protein contain sequences for insertion and cytotoxicity (Granum et al., 1981; Granum and Richardson, 1991). Amino acids 290-319 of the C-terminus contains the receptor binding region, and the receptor moiety *per se* is neither capable of insertion nor is it cytotoxic (Horiguchi et al., 1987; Hanna et al., 1989, 1992; Hanna and McClane, 1991). Other regions of the protein remain undefined and may be necessary for large complex formation. There has been considerable discussion as to whether a vaccine for CPE can be produced (Hanna et al., 1989, 1992; Mietzer et al., 1992). Purified CPE may present as many as five epitopes scattered throughout different regions of the enterotoxin. The monoclonal antibody 3C9 neutralizes CPE cytotoxicity by blocking receptor binding (Wnek et al., 1985). Since CPE C-terminal fragments are not cytotoxic and have neutralizing epitopes, they have potential for CPE vaccine construction. Immunity to *C. perfringens* type A FBI may also require a vaccine that would stimulate the production of high titers of secretory IgA anti-CPE in the intestinal lumen.

For a number of reasons, however, the production of a CPE vaccine may simply not be possible. In both human and animal studies, administration of CPE only induces transient immunity. Circulating antibodies to CPE are only present for a few weeks and there is no long term immunity (Bouvier-Colle et al., 1989; Hoffman

et al., 1987). Indeed there is some suggestion that the first toxicosis induces sensitivity to subsequent intoxication. This is similar in manner to the toxicosis presented after administration of *Staphylococcus aureus* enterotoxins (SEs). In one sense a lack of long-term immunity is not surprising since both groups of toxins are known to be superantigens. That CPE possess superantigenic activity is perhaps the most important finding about the nature of the molecule, since it may explain some of the unusual aspects of the toxin's activities, and the relationship CPE has with other non-foodborne diseases such as SIDS. It is very difficult, albeit impossible to generate long term immunity to a superantigen. Normal antigens activate 1:10,000 T cells while superantigens may generate 1:5. While this over stimulation of the immune system generates the release of high concentrations of cytokines, it does not allow for the generation of long term immunity since the immune response is so rapid. CPE-A over-stimulates a selected group of T cell receptors, namely Vß 6.9 and Vß 22 (Bowness et al., 1992), and out-competes SEA for binding sites.

Silent CPE Genes

Although microsequence variation of various *cpe* genes has been observed, the *cpe* ORF is generally highly conserved. The CPE sequence has little homology with other proteins, except for slight homology with a *C. botulinum* complexing protein, and a 5 amino acid sequence from the *Vibrio cholera* B subunit (Czeczulin et al., 1993, 1996; Hauser et al., 1994; Lindsay, 1996). Recent studies by Lindsay (1996)

indicates that a silent CPE gene is encoded within the intervening sequence of the Iota toxin gene from *C. perfringens* type E. There is 90.4% homology between the *cpe* gene sequence and that found associated with the iota toxin (Ula) (Lindsay, 1996). The potentially derived amino acid sequence from Ula shows 81.7% identity with CPE. Minor modifications (only single base changes) could increase the sequence identities to 96.8%. Lindsay (1996) calculated that the evolutionary relationship between the two sequences was 10 PAM (accepted point mutations per 100 residues) for the nucleic acid sequence (9.6 changes/100 nucleotides) and a PAM of 21 for the amino acid sequence (18.3 changes/100 amino acids). This argued that the evolutionary relationship was too close to simply be fortuitous. Comparison of the sequences upstream of the *cpe* and *pr-cpe* (IUa) genes showed similar regulatory Hpr sequences. This strengthened the evolutionary relationship between *pr-cpe* and *cpe*, and suggested that *cpe* is on a mobile genetic element which extends at least 500 nucleotides upstream of the *cpe* ORF (Lindsay, 1996).

CPE Intragastric Mechanisms of Action

The CPE mode of action *in vitro* and *in vivo* has few similarities with other bacterial toxins, thus it presents an unique toxicosis (McClane, 1994, 1997; Lindsay, 1996). Since CPE causes fluid and electrolyte losses in the GI tract of humans and animals, it has been classified as an enterotoxin (McDonel, 1986). Based on *in vivo* animal model studies, CPE appears to alter the fluid and electrolyte balance

compromising the villus integrity, thus breaking down the normal GI absorption and secretion mechanism, which is pathologically manifested as diarrhea (Sherman et al., 1994; Lindsay, 1996). Animal studies also show that CPE targets the small intestine with high affinity causing gross desquamation to the intestinal villi. Histopathological damage can occur very rapidly, thus CPE is also considered cytotoxic (McDonel and Duncan, 1975; McDonel, 1986; Sherman et al., 1994).

Characteristics of CPE Binding, Complex Formation and Insertion

The current model based on the extensive studies from McClane's lab is that CPEs action is a multi-step process with four events. Briefly, CPE first binds to a 50 kDa membrane protein creating a 90 kDa small complex. Second, the structural change of CPE contained in the small complex, which results from either CPE or small complex insertion into the cellular membrane, or from small complex conformational changes. Third, the formation of a 160 kDa large complex resulting from the small complex binding to a 70 kDa membrane protein (Wnek and McClane, 1989; McClane, 1997). The fourth and final step is the loss of plasma membrane permeability properties, caused by either the large complex directly serving as a pore or simply by directly affecting membrane permeability (Wieckowski et al., 1994, McClane, 1994, 1997; Czeczulin et al., 1996).

The CPE receptor(s) is found in several different cell types of mammalian species and due to it's broad distribution the receptor is thought to have an important

physiological role, but not be essential for cell viability (McDonel and McClane, 1979; McDonel, 1980; Wnek and McClane, 1986; McClane et al., 1988b; Sugii and Horiguchi, 1988; McClane, 1994). Receptors is in plural since there is some disagreement as to whether more than one receptor exists. Kinetic studies from different labs found contradictory results (McDonel, 1986; McClane, 1994). Affinity chromatography studies of brush border membranes (BBM) and Vero cells strongly suggest two proteinaceous CPE receptors of 50 kDa and 70 kDa (Wnek and McClane, 1983; Wnek et al., 1985; Sugii and Horiguchi, 1988). Additional support for the 50 kDa protein came from immunoprecipitation analysis of a 90 kDa CPE-mammalian protein complex (small complex) that consisted stoichiometrically of one 50 kDa membrane protein and one CPE molecule (Wieckowski et al., 1994). The 160 kDa protein was determined from molecular weight analysis of the large protein complex indicating the complex was stoichiometrically composed of one 50 KDa protein, one CPE molecule and a 70 kDa protein. CPE binding is receptor specific and saturable (10^6 CPE receptors per cell). Binding occurs rapidly (15-30 min) and in one tissue culture system binding appeared to be temperature sensitive (CPE binding at 4°C is lower than at 37°C) (McDonel and Duncan, 1975; McClane et al., 1988b; McClane, 1994; Wieckowski et al., 1994).

The action mode of many membrane-active toxins, including CPE, often involves plasma membrane-permeability imbalances through toxin insertion into the lipid bilayer (McDonel, 1980; McClane, 1994). The McClane model suggests that

after CPE binds to receptors it remains inserted in the lipid bilayer, toxin is neither dissociated nor internalized (McClane, 1994). Binding to receptor(s) is a two-step process during which CPE acquires amphiphilic capabilities (Wieckowski et al., 1994). As previously noted, binding is irreversible, protease resistant and possibly temperature dependent (McClane et al., 1988b; McClane, 1994). CPE is inserted into the membrane after binding and is entrapped within the lipid bilayer. Although the CPE-small complex undergoes conformational changes, it is unknown whether the entire CPE-small complex or CPE alone remains entrapped (Czeczulin et al., 1996). Unlike small complex formation, CPE-large complex formation is temperature dependent. Large complex is formed above 24°C but not at 4°C, thus at 4°C CPE cytotoxicity is blocked. Cytotoxicity can be unblocked by transfer to higher temperatures. Although the 70 kDa protein is part of the large complex, it is unknown whether the molecule is a receptor. Possibly it is simply a functional membrane protein brought into close contact with the CPE-small complex through stearic attraction, CPE insertion into lipid bilayer, or conformational charge in the small complex (Kokai-Kun and McClane, 1996).

Large complex formation is thought to either directly affect plasma membrane permeability (influx and efflux) by functioning as a membrane "pore" (ion-permeable channel) or by interfering with membrane pump regulation via continuous pump activation (Sugimoto et al., 1988; Czeczulin et al., 1996; Kokai-Kun and McClane, 1996). The permeability changes may develop within 5 minutes, and restrict passage

to ions and small amino acids (less than 200 Daltons) that transit through membrane lesions of about 0.5 nm^2 in size (McDonel and McClane, 1979; McDonel, 1986; McClane, 1994; Czeczulin et al., 1996; Kokai-Kun and McClane, 1996). Since eukaryotic cells have a lower intracellular ion concentration than the external medium in order to maintain a normal colloid-osmotic equilibrium (McClane, 1994), permeability changes allow for a rapid ion influx into the cell cytoplasm. This initial influx is calcium ion dependent, requiring elevated intracellular levels of Ca^{2+} ions. High levels of calcium ions may lead to collapse of the cytoskeleton (McClane, 1994). A rapid influx of small molecules causes the plasma membrane to "stretch" facilitating the additional influx of macromolecules > 5 kDa. This leads to gross osmotic changes and cell destabilization, often seen as membrane blebs. Loss of essential amino acids causes secondary effects of inhibition of DNA, RNA and protein synthesis, and thus the cells become nonviable (McClane and McDonel, 1979; McDonel, 1986; McClane et al., 1988a; Hulkower et al., 1989).

CPE and Medicine

Although the above described CPE-induced changes were elucidated from *in vitro* cell studies, it is believed that the same effects occur *in vivo* during CPE induced FBI. CPE affects intestinal epithelial cells in a similar fashion. Villous epithelial cells present small molecule permeability changes, disruption of normal villus integrity and function, morphologic damage, cell lysis and net secretion of fluids and

electrolytes (McClane, 1994). The ability of CPEs to kill a wide variety of cells both *in vivo* and *in vitro* has also led to the evaluation of CPE-86 as anti-neoplastic agent (Lindsay, 1996). Preliminary studies showed that CPE-86 was destructive to Lewis lung carcinoma cells *in vivo* and neoplastic cell lines (P388, B16-F1, and L110). The mechanisms of neoplastic cell death were the same as in Vero cells with membrane permeability changes, bleb formation, inhibition of nucleic acid and protein synthesis, and subsequent cell death. Bacterial toxins have a great potential as anti-cancer drugs (Pastan et al., 1995; Lindsay, 1996), and several plant and bacterial toxins (ricin, *Pseudomonas* exotoxin A) have previously been suggested as therapeutic agents for cancer treatment via linkage to cell specific antibodies (magic bullets). Although the data with CPE-86 is preliminary it suggests that the *C. perfringens* CPEs have a potential use in medicine.

Activation of CPE

CPE causes dose dependent death in mice where mice die quietly with symptoms of respiratory interference and shock, while *in vitro*, CPE induces dose dependent morphological damage, inhibition of nucleic acid synthesis, modulation of membrane transport, lysis and cell death in Vero cells (McClane and McDonel, 1979; Mach and Lindsay, 1994; Lindsay, 1996). Human fetal ileal cells (FI) (ATCC CCL241) are resistant to the action of CPE, even at toxin levels of 1 μg/well. The mechanism of this resistance is unknown, and it can be considered very strange that

cells from the actual tissue affected *in vivo* by CPE should not be affected *in vitro*. A possible explanation is that since the FI cells are derived from fetal tissue they are likely undifferentiated. In this state they are not susceptible, and require differentiation to become susceptible.

Studies by McClane et al., (1987) and Mach and Lindsay (1994) indicated that the activity of CPE can be dramatically exacerbated both *in vivo* and *in vitro* by the presence of interferon-gamma (IFN-γ). *In vivo*, the presence of IFN-γ can decrease the LD_{50} 1,000 fold and reduce the time to death 360 fold. *In vitro* both Vero and FI cells either pretreated with IFN-γ then CPE, or IFN-γ combined with CPE showed dramatic sensitization with a several log fold increase in CPE activity.

Enhancement of CPE Activity and Human Non-Foodborne Disease

Both groups proposed that enhancement of activity likely resulted from IFN-γ sensitizing cells to the action of CPE. *In vitro*, IFN-γ possibly acts by sensitizing cells to the action of CPE resulting in death, possibly by the same mechanisms (inhibition of protein synthesis and destruction of the cell membrane) but at lower toxin concentrations. Mach and Lindsay (1994) and Lindsay et al., (1994) suggested that these observations assisted in the formulation of a toxico-hypothesis for the sudden infant death syndrome (SIDS). The observation that a majority of victims have infections in the two weeks prior to death is known (Morris, 1987; Murrell et al., 1987; Murrell et al., 1992). Viruses, bacteria and bacterial toxins are all known

inducers of IFNs (Collier and Kaplan, 1985; Pestka et al., 1987; Ijzermans and Marquet, 1989; Baron et al., 1991; Chonmaitree and Baron, 1991). Therefore, there may be a link between prior infection increasing IFN levels, sensitization to bacterial toxins and SIDS. Although studies conducted over 15 years ago in relation to viraemia and SIDS showed no evidence of a systemic viral infection or elevated interferon at death (Ray and Hebestreit, 1971; Seto and Carver, 1978). It is possible that increased circulating IFN levels might not have been observed, since the immune response to the initial antigen had abated. Alternately, the tissue-cell-methodology for detecting IFNs may not have been very sensitive. Howatson (1992) recently examined the relationship between viral infection and the production of IFN-α. He concluded that the abnormal presence of IFN-α in neurones of the medulla of the brain stem suggested that it was premature to discount a viral hypothesis for some proportion of SIDS cases. Jakeman et al., (1991) recently showed that the toxicity of several bacterial toxins could be significantly exacerbated in infant ferrets by a previous infection with influenza virus. These authors speculated a role in SIDS by some mechanism(s), including an enhancement of cell permeability which may allow increased or more rapid uptake of a toxin, resulting in death by the same mechanism but at lower concentrations. Mach and Lindsay (1994) argued that by extension, it is likely that the same situation occurs in human infants. They proposed that a window of vulnerability occurs in the life of some infants due to immunological immaturity, which predisposes them to infection. In the weeks prior to death, these

infants suffer from an infection which induces the synthesis of IFNs, sensitizing the infant to a later albeit more virulent infection which may act as a trigger for sudden death. See the following section for further discussion of the CPE toxico/SIDS hypothesis.

Role of CPE in Non-Foodborne Diseases (SIDS)

CPE has recently been implicated in a very unusual disease state, the sudden infant death syndrome (SIDS). SIDS is defined as the " sudden death of an infant from one month to one year of age, which remains unexplained after a complete post-mortem examination, including an investigation of the death scene and a review of the case history" (Hoffman et al., 1988; Zylke, 1989). In the United States SIDS claims the lives of 6-7,000 infants (1/1,000 live births) (Willinger, 1989) and remains the number one cause of post-neonatal infant mortality (Lindsay, 1996). While a large number of theories have been proposed for SIDS, the reason for this large number of infant deaths remains unresolved (Staton, 1980; Valdes-Dapena, 1980; Thach et al., 1988; Verrier and Kirby, 1988; Spika et al.,1989; Wilkinson, 1992). There are many epidemiological indices found in SIDS victims, however, the most striking are the age at death, and that $>$ 85% of SIDS victims were ill in the two weeks prior to death. It has been suggested that some infants present a "window of vulnerability" where physiological discrepancies or abnormalities make them susceptible (Stephens, 1990; Lindsay et al., 1992, 1993; Murrell et al., 1993; Murrell et al., 1994; Lindsay,

1996). The nature of these abnormalities are as yet unresolved, however, they may possibly explain why SIDS fatalities occur within such a narrow time frame (Willinger, 1989). Although premature infants are more susceptible to SIDS, prematurity is not a determinant, since gestational age is not related to age at death (Buck et al., 1988; Grether and Schulman, 1989). This might suggest that a common factor or factors are responsible for, or triggers the biochemical changes that lead to death.

Common pathological indices observed at autopsy include thymic petechiae (pinpoint hemorrhages) and patchy pulmonary edema, muscle fiber necrosis of the diaphragm with a histopathology suggesting hypoxia, astrogliosis of the brainstem, leukomalacia (Jones and Weston, 1976; Valdes-Dapena, 1983; Krous, 1984; Guilian et al., 1987; Beckwith, 1988; Hollander, 1988; Gillan et al., 1989; Guntheroth, 1989; Kariks, 1989; Bruce and Becker, 1992). It has been suggested that these indices might result from or be caused by infections. It should be stressed that the presence of a particular pathogen within an infant is not predictive of a disease state, or SIDS. Most infants have infections during their first year of life, yet only a small percentage die from SIDS (Arnon, 1983; Bettelheim et al., 1990; Blackwell et al., 1994, 1995). Therefore it has been suggested by several authors that a small sub-population of infants are predisposed to SIDS and when the correct "conditions" occur death is a likely sequelae.

CHAPTER 3
DETECTION OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN AFTER *IN VITRO* BINDING TO MURINE TISSUES

Introduction

In humans, a *Clostridium perfringens* foodborne illness (FBI) occurs 6-12 hours after ingestion of vegetative cells. Upon entering the host *C. perfringens* responds to changes in environmental stress by initiating the sporulation cycle with concomitant production of enterotoxin (CPE), and subsequent induction of FBI. In the small intestine, CPE utilizes a unique mechanism of action to directly affect the plasma membrane of cells leading to inhibition of macromolecular synthesis, morphological damage and cell lysis (cytotoxicity), and fluid loss (enterotoxigenicity). Desquamation of villous cells allows CPE to be absorbed and systemically distributed throughout the body, causing various pathophysiological (neurotoxigenic) and immune responses (superantigenic), which may lead to severe illness or death.

In order to study the pathophysiological and immune responses generated by CPE, the whole body distribution of toxin after absorption must be determined, which was the overall goal of this dissertation. To fully elucidate toxin distribution, administration had to be performed both intraperitoneally, and through the natural port of entry, the digestive tract (intragastrically). The aim of the work described in

this chapter was to determine, using *in vitro* techniques and the murine model, which tissues had the ability to bind intact CPE molecules.

Materials and Methods

Murine Model

During the last 25 years there has been a large number of animal species used as model systems to study the action of CPE. Dr. Lindsay's lab has placed a particular emphasis on mice for a number of reasons. First, the species use in a wide range of published toxicological studies, and the relative inexpensive cost of purchasing and maintaining large numbers of animals. Second, large numbers of animals that are genetically similar increases the validity of results. Third, mice have been extensively used by other researchers to examine the effects of bacterial toxins. Fourth, the mode of action, symptoms and pathophysiological changes caused by CPE in the murine model strongly mimic those observed in human cases of CPE toxicosis. Taking all these factors into consideration, we believe that the mouse is an excellent model, and its use can be justified.

C. perfringens Enterotoxin (CPE)

Purified, freeze dried CPE was gratefully obtained from Dr. Bruce McClane, University of Pittsburgh. For administration, toxin was resuspended in phosphate buffered saline-Tween (PBS-TW) (0.15 M NaCl, 0.01 M Na_2HPO_4, 0.01 M

NaH_2PO_4, and 0.2% Tween 20, pH 7.2) and standardized to a final concentration of 1 $\mu g/\mu l$ of protein. Protein determinations were made by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Preparation of Antisera to CPE

Three milligrams of purified CPE were supplied as antigens. Individual 4-5 kg New Zealand White rabbits were used to generate antisera. One milliliter of Freud's Complete adjuvant was emulsified with 1 mg (300 μl) of the antigen and 5 injections (3 intradermal, 1 subcutaneous, 1 intramuscular) were administered to the rabbits. The injections were repeated using 1 mg of antigen and 1 ml of Freud's Incomplete adjuvant 30 days later and again 14 days after that. To monitor the titre, test bleeds from the ear vein were done every 7 days after an injection. After the third series of injections, the rabbit was anesthetized with Ketamine and Rompun and bled by cardiac puncture. Serum was separated by centrifugation and stored at -20°C.

Biotinylation of Antisera

Antisera to CPE was biotinylated using NHS-LC-Biotin (Pierce, Rockford, IL). One hundred microliters of 0.05 M bicarbonate buffer, pH 8.5, was added to 2 mg of antisera. NHS-LC-biotin (0.04 mg) was added to the antisera and incubated on ice with mixing every 20 minutes for 2 hours. Unreacted biotin was removed by the addition of PBS to a total volume of 1 ml followed by dialysis in 6-8,000 wt cut off

dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) against two 500 ml volumes of PBS for 16 hours. The biotin labeled antisera was aliquoted and stored at -20°C.

Animals

Three week old (12-13g) male Swiss Webster (SW) mice used in this study were obtained from Harlan-Sprague Dawley, through the University's Department of Animal Resources. The Department of Animal Resources is an IACUC Veterinary controlled facility. Animal Resources (AR) order, install, raise and maintain the animals within the Food Science and Human Nutrition (FSHN) Departmental Animal Facility as prescribed by the University of Florida IACUC. Animals are kept on a 12/12 light-dark cycle at 25°C and are examined on a daily basis by AR who also change bedding and provide food and water. All animal studies were performed within the FSHN Animal Facility.

Animal Tissue Preparation

Mice were allowed to reach body weights of between 15-18 g before experimental use. This was usually 4-6 days after delivery, which allowed time for the animals to become accustomed to their surroundings and being handled, thus reducing any contributing stress factors. Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Mice were dissected and all organs

(brain, thymus, heart, lung, liver, kidney and small bowel) were isolated, washed in PBS-TW, weighed and transferred to sterile 15 ml polypropylene tubes. Three milliliters of PBS-TW were immediately added and each organ was homogenized and disrupted on ice using a Polytron (Brinkmann Instruments) three times for 15 seconds. All individual organs from each mouse were kept separately in polypropylene tubes, to which CPE resuspended in PBS-TW was added at a standardized concentration, vortexed and allowed to bind.

CPE:Tissue Binding

To determine the level of CPE binding to tissue from each organ, five separate experiments (E1-E5) were performed, each requiring 8 mice. In four experiments the toxin concentration varied from 40 ng (E1) to 80 ng (E2), 1 μg (E3) and 2.5 μg (E4) of CPE/ml of tissue homogenate, with incubation for 1 hour at 27°C (lab controlled to this temperature). In the fifth experiment (E5) the CPE concentration was 2.5 μg/ml tissue homogenate, with incubation at 4°C for 4 hours. During the incubation period, samples were gently vortexed every 10 minutes to ensure complete distribution of toxin and tissue. Each experiment contained an equivalent number of negative controls, that is, tissues treated with only PBS-TW and no (zero) CPE. After incubation, a 1 ml sample from each individual organ was removed, and the remaining homogenate was stored at -70°C. The 1 ml isolated supernatant sample

was centrifuged at 12,000 x g for 30 minutes at 4°C, and the supernatant collected and stored at -70°C.

Enzyme Linked Immunosorbent Assay (ELISA)

The ELISAs were conducted using the methodology described by Crowther (1995). Before the binding experiments were performed, extensive preliminary studies (concentration vs concentration analysis) were undertaken to determine the optimum levels and incubation times for each of the ELISA components and steps. A 200 μl supernatant sample (At: defined as soluble protein-antigens) from each of the tissues was thoroughly mixed with 200 μl of coating buffer (CB: 1.5 mM Na_2CO_3, 3.3 mM $NaHCO_3$, 3 mM NaN_3). Triplicate 100 μl aliquots were added to individual wells of a polystyrene 96 well Immunolon™ 2 flat bottom plate (Dynatech Laboratories, Chantilly, VA), and allowed to passively adsorb for 16 hours at 4°C. Any unbound sample was then aspirated, and each well was washed five times (X 5) with 100 μl PBS-TW. Primary polyclonal antibody (1°Ab) to CPE previously diluted to $1/10^{-3}$ in PBS-TW was added to each well (100 μl/well) and allowed to adsorb for 16 hours at 4°C. Any unbound 1°Ab was then aspirated, and each well was washed X 5 with 100 μl PBS-TW. Secondary (2°Ab) goat-anti rabbit IgG antibody labeled with the enzyme alkaline phosphatase (AP) (Sigma Chemical Co., St. Louis, MO) diluted to $1/10^{-3}$ in PBS-TW was added to each well (100 μl/well) and allowed to adsorb for 2 hours at 27°C. Wells were then aspirated and washed as described

previously. The At:1°Ab:2°Ab-AP complex was detected by the addition of 100 μl of alkaline phosphatase buffer (50 mM Na_2CO_3, 50 mM $NaCO_3$, 0.5 mM $MgCl_2$) containing 100 μg of p-nitrophenyl phosphate (Sigma Chemical Co.). The enzyme reaction was stopped after 1 hour by the addition of 100 μl of 1.0 N NaOH. Presumptive CPE positive samples are indicated by a sample color change from clear to yellow, which was quantitated by spectrophotometric analysis at 405 nm (Bio-Rad ELISA reader, model 2550).

Western Immunoblot Analysis

Tissue proteins and any CPE contained in the supernatant were separated by polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) using Bio-Rad Mini-Protean II slab gels and the buffered system of Laemmli (1970). Stacking and separating gels were 4.0% and 10% polyacrylamide, respectively. Prestained protein molecular weight markers (27-180 kDa) were obtained from Sigma Chemical Co. Gels were stained with 0.15% Coomassie Brilliant Blue R-250 overnight after being used for Western immunoblots. After soaking in transfer buffer (0.192 M glycine, 0.025 M Tris, 20% methanol) for 30 minutes, gels were immunoblotted using a Bio-Rad mini Trans-blot electrophoretic transfer cell and nitrocellulose membranes (Stratagene, La Jolla, CA) according to manufacturers directions. After transfer, the membrane was blocked with PBS containing 0.1% Tween 20 and 7.0% casein for 1 hour. The membrane was drained and soaked for

16 hours in a 10^{-3} dilution of biotinylated antisera in blocking buffer. The membrane was then washed and soaked in a 1 mg/ml solution of strepavidin (Sigma Chemical Co.) for 2 hours. After a second washing step with PBS-TW the membrane was soaked and rinsed 3 times for 5 minutes with substrate buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). The protein bands were made visible by soaking the membrane in 10 ml of substrate buffer containing 0.1 mg/ml of nitroblue tetrazolium and 0.05 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate for 30 minutes at 4°C.

Statistical Analysis and Interpretation

Statistical analysis of ELISA readings was performed using an one-way analysis of variance (ANOVA) with the STATISTICA for Windows software program, release 4.5 (Copyright© StatSoft, Inc. 1993). When a significant difference was found ($p < 0.05$), Post hoc comparisons were done by using Tukey's honest significant difference (HSD) test, choosing an alpha level for critical ranges set at ($\alpha = 0.05$).

Results and Discussion

ELISA Data Interpretation and Difficulties

The aim of these experiments was to determine, using *in vitro* techniques, to which tissues (organs) within the mouse the CPE molecule had the highest affinity. Previous studies (Wnek and McClane, 1989; McClane and Wnek, 1990) have

indicated that one CPE molecule binds to a single 50 kDa receptor (R1) on the cell surface to form the CPE-R1 "small complex". Formation of small complex occurs at both temperatures 4°C and 27°C. Large complex formation occurs when the hydrophobic portion (N-terminal:toxic) of CPE is internalized and interacts with a 70 kDa protein (receptor R2) to form CPE-R1-R2. This interaction (large complex formation) is irreversible and does not occur at 4°C.

Several definitions were made for the ELISA results. First, an ELISA was considered statistically positive after performing an ANOVA ($p < 0.05$) and a Post-hoc comparison (HSD test, $\alpha = 0.05$). The positive ELISA ($\alpha = 0.05$) indicated that after the tissue-CPE interaction was completed, the isolated supernatant still contained free CPE molecules. Second, an ELISA was considered statistically negative after performing an ANOVA and a Post-hoc comparison ($\alpha = 0.05$) where no significant difference was detected between control and CPE treated tissues. Thus it appears that a given tissue supernatant contained a greater number of R1 receptors than toxin which bound all available CPE molecules. Third, that if the experiments were conducted with fresh organ tissues at 27°C, then CPE binding to receptor R1 would form the small complex (CPE-R1). This small complex would, within the 1 hour incubation period conformationally change and interact with receptor R2 to form the large complex (CPE-R1-R2). Since we were using polyclonal antibodies to CPE, a number of epitopes on the CPE molecule were available for interaction (binding). We simply did not know whether the interaction of CPE with R1 on the membrane

left any regions of the toxin molecule available for interaction with the 1°Ab. This is not an unlikely situation since the N-terminal hydrophobic portion of CPE does not appear to bind to R1, and thus could be available. However, since the binding of CPE to R1 is temperature dependent and rapid, we believed that if CPE did bind to R1 to form the small complex, then large complex formation would be relatively rapid, and thus no portion of the CPE molecule would be available to bind to the 1°Ab. Additionally, if the amount of tissue from a specific organ was kept constant and the amount of toxin varied, it might be possible to approximate CPE saturation levels for a specific tissue. That is, since the molecular weight of CPE is known, it was possible to calculate the number of CPE molecules/mg protein, and thus the number of R1 receptors/organ.

Initially there were some difficulties in conducting the ELISAs, particularly for liver and kidney tissues which had a higher degree of non-specific binding. This was possibly a function of some tissues having both a higher amount and more varied number of tissue proteins, which may have caused some stearic hindrance. Alternately some proteins may have had a higher non-specific affinity for the 1°Ab, caused by conserved protein regions common to both CPE and tissue proteins, resulting in stearic interference. Fortunately, most of the non-specific binding problems were resolved by using purified 1°Ab, and additional blocking steps which significantly reduced background interference. Despite these problems, we believe the ELISA results are valid for all tissues.

Table 3.1 Detection of unbound CPE by ELISA in murine organ tissues after *in vitro* interaction.

	CPE concentration and binding conditions				
Organ	40 ng	80 ng	1.0 μg	2.5 μg/27°C	2.5μg/4°C
Brain	--	--	--	++	++
Thymus	--	--	--	++	++
Heart	--	--	++	++	++
Lung	--	--	++	++	++
Liver	--	--	--	--	--
Kidney	--	--	--	--	--
Bowel	--	--	--	--	--

Binding conditions: 40 ng/ml CPE at 27°C for 1 hour
80 ng/ml CPE at 27°C for 1 hour
1.0 μg/ml CPE at 27°C for 1 hour
2.5 μg/ml CPE at 27°C for 1 hour
2.5 μg/ml CPE at 4°C for 4 hours

(--) = no free CPE detected [$\alpha = 0.05$]
(++) = free CPE detected [$\alpha = 0.05$]

CPE Binding

A summary of the five experiments described in the materials and methods is shown in Table 3.1. In experiments E1 (40 ng/ml) and E2 (80 ng/ml) no free CPE was observed in the tissue supernatant from any mouse organ. This suggested that neither of these toxin levels saturated the R1 sites in any organ tissue. The molecular weight (MW) of a compound is the sum of the atomic weights of the atoms in the molecule. It is the number of grams containing Avagadro's number of molecules (6.022×10^{23}) (a mole is Avagadro's number of molecules). Thus, since the MW of the CPE molecule is 35,317 (Czeczulin et al 1993) then the minimum number of R1 receptors in each of the organ tissues examined in this study was $> 8.5 \times 10^{9}$ /mg.

Experiment E3 examined a 12.5 fold increase in CPE to 1,000 ng/ml. Results indicated that at this level the heart and lung showed free CPE at a significance level of $\alpha = 0.05$. This suggested that the receptor-saturation threshold for CPE in the these organs was between 80-1,000 ng/ml, and the number of R1 receptors per mg of tissue was between $> 8.5 \times 10^{9}$ /mg and $< 1.5 \times 10^{11}$ /mg. No free CPE was detected with the brain, thymus, liver, kidney and bowel. Experiment 4 examined a 2.5 fold increase in CPE from E3, and a 32 fold increase over E2. The results obtained were consistent with E3 indicating that 2,500 ng/ml of CPE saturated R1 receptors in thymus, heart, lung and brain tissue. Receptor-saturation threshold for CPE in the brain and thymus was between 1,000-2,500 ng/ml, and the number of R1 receptors per mg of tissue was between $> 4.3 \times 10^{10}$ /mg and $< 1.5 \times 10^{11}$ /mg for

the brain and between $> 2.0 \times 10^{11}$ /mg and $< 5.0 \times 10^{11}$ /mg for the thymus. No free CPE was found with liver, kidney and bowel tissues indicating that the number of CPE R1-receptors in these tissues was $> 1.5 \times 10^{11}$ /mg tissue. These results confirm previous conclusions by McDonel (1980): first, that the receptor saturation threshold for liver, kidney and bowel tissues was $> 1.5 \times 10^{11}$/mg of tissue protein, and there appeared to be no difference between the number of CPE-receptors between these organ tissues. Second, he also considered that competitive or inhibitory factor(s) released from the brain homogenate into the reaction buffer prevented significant CPE-receptor binding to this specific tissue. In this present study significant binding of CPE to brain tissue was found, which contradicts McDonel (1980). However, it should be noted that in this current study the methods used were far more sensitive and the binding technique was different. McDonel manipulated the tissue through a greater number of steps which may have caused the release of the "inhibitory" CPE-binding factor he suggested.

One of the initial assumptions was that even if CPE bound to R1, the small complex formed might still allow additional binding of 1°Ab to any available regions of the CPE molecule. We argued however, that under our experimental conditions any small complex formed would conformationally change and become large complex very rapidly. Thus 1°Ab binding to small complex was not an issue. Small complex formation is temperature independent, however, large complex formation is temperature dependent. In E5 we examined whether saturating levels of CPE (2,500

ng/ml) gave the same results at 4°C when compared to using 2,500 ng/ml CPE at 27°C (E4). Table 3.1 shows that indeed the results from E4 and E5 are directly comparable, where in both experiments free toxin is only found in the supernatants of heart, lung, thymus and brain tissues, but not in liver, kidney and bowel.

There are several possible alternatives. If McClane and Wnek (1990) are correct and small complex formation is temperature independent and large complex formation temperature dependent, it simply makes no difference, since no free toxin is found in some tissues. That is, in liver, kidney and bowel tissues small complex formation does not appear to allow any residual 1°Ab binding to CPE. The small complex must simply be in an unavailable conformational state. Although it cannot be equivocally stated that this situation also occurs with heart, lung, thymus and brain tissues, it would seem reasonable to assume that CPE binding in these tissues is no different than in liver, kidney and bowel.

Western Immunoblots were performed to determine if the technique could distinguish between control and CPE treated tissue. Only lung tissue was examined since this organ bound a significant amount of toxin, yet had a low level of interfering soluble proteins compared to liver and kidney. Preliminary studies indicated that with the Western immunoblot technique, the lower limit of CPE detection either as pure toxin or unbound CPE was 50 ng/ml. Results shown in Figure 3.1 lane C indicated that control (untreated) tissue showed no positive bands, indicating that contaminating tissue proteins from the lung did not bind non-specifically to the 1°Ab. When CPE

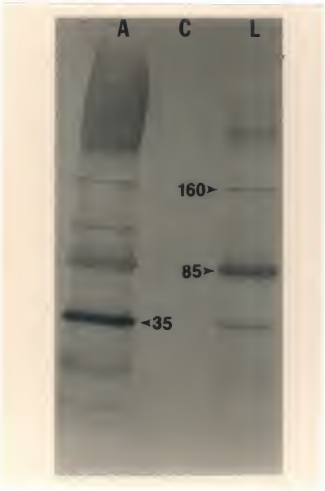


Figure 3.1 Western immunoblot of lung tissue: CPE, lung tissue without CPE, and pure CPE detected with a 10^{-3} dilution of antisera to CPE. The amount of protein (CPE) in each sample is noted in parenthesis. Lane A: purified CPE (28 μg); lane C: lung tissue supernatant without CPE (0 μg); lane L: lung tissue supernatant with CPE (100 ng).

was in excess to the receptors within the lung (see Table 3.1 treatment 1.0 μg, and Figure 3.1 lane L) free toxin was clearly detected as a M_r 35,000 band (lane L) exactly at the same position as pure CPE (lane A). Strong antibody positive bands were also detected at approximately M_r 87,000 (small complex) and 160 kDa (large complex) (lane L).

These *in vitro* binding studies suggested that the liver, kidney and bowel had the highest number of CPE receptors within their tissue ($> 4.5 \times 10^{13}$ receptors/ organ), followed by brain and thymus ($\leq 4.5 \times 10^{13}$ receptors/organ), and heart and lung ($\leq 1.7 \times 10^{13}$ receptors/organ). These results are not unexpected since introduction of CPE to the body occurs via the gastrointestinal tract, and metabolism and detoxification of CPE likely occurs via the kidneys and liver. Indeed Skjelkvale et al. (1980) in his studies with ^{125}I-labeled CPE suggested that the kidney was a target organ for CPE binding and that this organ contained the highest level of CPE receptors. The studies conducted herein, confirm that the kidney is an organ that contains a large number of CPE receptors, however, there is no indication that this organ is the main target. Skjelkvale et al. (1980) suggested that CPE is metabolized and expelled from the body within urine, which would make the kidney an organ of focus. However, further *in vivo* studies are required to confirm this. The liver and small bowel would also be organs of focus since cells from these tissues are highly susceptible to the action of CPE (Lindsay and Dennison, 1986a, 1986b; Kokai-Kuhn and McClane, 1996; Mach and Lindsay 1997). Although our data strongly suggest

that liver, kidney and bowel tissues contain the highest number of receptors, and require very high levels of CPE to reach receptor saturation, the results do not necessarily translate to the *in vivo* model. That is, these organs may be the main focus of CPE distribution but not necessarily CPE activity *in vivo*. It is possible that low levels of CPE within a specific tissue have a more dramatic effect on the host, than high levels of CPE in another. For example, high levels of CPE in the bowel may be enterotoxigenic and cytotoxic causing diarrhea, fluid loss and tissue desquamation. However, low level CPE binding and activity in brain and lung tissue may affect neurologic and respiratory status. Additionally, CPE binding to heart tissue may have cardiotrophic effects. Thus, enterotoxigenicity may be transient, but alteration in neurologic, respiratory or cardiac status may be lethal. Chapters 4 and 5 detail our studies to determine the *in vivo* distribution of CPE after intraperitoneal and intragastric administration and the consequential effects.

CHAPTER 4
DETECTION AND DISTRIBUTION OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN AFTER *IN VIVO* INTRAPERITONEAL ADMINISTRATION INTO SWISS WEBSTER MICE

Introduction

McDonel (1980) indicated that *in vitro*, CPE binds with different specificities to various organ tissues. Results suggested that liver and kidney tissues contained the largest number of CPE receptors, and that both organs were specific sites of CPE attack that required > 0.5 μg of CPE/mg of tissue to reach receptor saturation. There is however, an anomaly in McDonel's supposition. The data suggested that to reach complete saturation of liver and kidney tissues *in vivo* required lethal levels of CPE to be administered and absorbed, an unlikely circumstance in most CPE induced foodborne illnesses. Skjelkvale et al. (1980) using the murine model attempted to determine the distribution and levels of CPE binding after intravenous (IV) administration of radioiodinated CPE. Results suggested that the liver and kidneys were specific organs of CPE accumulation and attack, and that a major fraction of CPE was rapidly metabolized and excreted in urine. This apparently confirmed McDonel's findings, however, alternative explanations are possible. First, decay of radiolabeled toxin could have spread radioactivity to organs where CPE was not apparent, leading to false positives. Second, CPE was not administered through its

natural port of entry, that is intragastrically (IG). It is not known whether CPE administered both IP and IG give similar results. It could be argued however, that this second point is moot, since Tsai and Riemann (1975a) showed that CPE orally administered to mice was present in blood within minutes. Thus, IG and IP administration may be similar, however, CPE is known to be structurally altered by proteolytic enzymes in the small bowel before systemic absorption (Granum and Richardson, 1991). Third, CPE distribution in mice was only monitored during the two hours after IV administration, and only at a single CPE dose. It is possible that there are various CPE distribution patterns which are toxin dose and time dependent. This chapter describes the approaches to determine the organ/tissue distribution of varying doses of non-labelled CPE toxin after IP administration into Swiss Webster mice.

Materials and Methods

CPE-Toxin and Antisera

Freeze dried CPE obtained from Dr. Bruce McClane was prepared for IP administration as described in Chapter 3. The biological activity (specific activity) of preparations was examined before use by Vero cell analysis (Mach and Lindsay, 1994) and was standardized to 4,000 EU/μl toxin. The methods to produce antisera to CPE are described in Chapter 3.

Animal preparation and IP enterotoxin administration

Animals were purchased and maintained as described in Chapter 3. As all animals were bred from the same line and obtained from the same source it was assumed that they were genetically similar. Gross examination indicated no apparent phenotypic differences. When animals weighed 15-18 g (25-30 days old), they were randomized and grouped six/cage. To reduce any administration differences, one person (Keller) held the mice in the correct alignment, while another (Lindsay) measured the correct volume and performed the administration. Animals were administered CPE IP in the left side of the peritoneal cavity, using a 1 ml tuberculin syringe and a 27 gauge needle. After injection animals were returned to their respective cages and monitored every 15 minutes.

Study 1a: CPE Concentration

This study was performed to determine the following: i. the CPE concentration at a time which caused death within 72 hours; ii. the CPE concentration at which the pathophysiological changes observed during the toxicosis could be strongly predicted without interference from cases of random death within the treated mouse population. These data would then allow animal necropsy and tissue sampling with reliability.

CPE was administered IP at various levels ranging from 0.1 to 5 mouse lethal doses (MLD): nonlethal 0.1 MLD (0.5 μg/250 μl) to 0.25 MLD (1.25 μg/250 μl); sublethal 0.5 MLD (2.5 μg/250 μl) to 1.0 MLD (5.0 μg/250 μl); lethal 2.0 MLD

(10.0 μg/250 μl) to 5.0 MLD (50.0 μg/250 μl); and control: 250 μl of PBS. Twelve mice were used at each treatment level, and 3 control mice were administered PBS. Any pathophysiological changes were monitored every 15 minutes from T_0 (immediately after administration) to T_{72} hours, or time to death, which ever came first. Mice were necropsied and all organs (brain, thymus, lung, heart, liver, kidney, and small bowel) were isolated, washed in PBS-TW, weighed and transferred to sterile 15 ml polypropylene tubes and stored at -70°C for later analysis. Blood and urine (where possible) were also collected. Before storage, blood samples were gently centrifuged at 200 x g to collect serum. Both serum and urine were stored at -70°C for analysis.

Study 1b: CPE Distribution vs Time

Based on data obtained in Study 1a (see Results and Discussion for detailed data) a CPE concentration of 10 μg CPE/250 μl PBS was chosen for IP administration in the distribution versus time study. Twenty four animals were prepared, and administered CPE IP in a single dose as described above. Six similar weighted animals were administered PBS as controls. At six time intervals 0.25, 1, 2, 3, 4, and 5 hours, four animals were randomly chosen from the toxin administered group and one from the control group and euthanized. Mice were necropsied and all organs (brain, thymus, lung, heart, liver, kidney, and small bowel) were collected as described above. Blood and urine (where possible) were also collected. Before

storage, blood samples were gently centrifuged at 200 x g to remove red blood cells, and the serum collected and stored at -70°C, as was any urine sample.

Immunological Methods

To prepare organ tissue samples for ELISA, 3 ml of PBS-TW was added and each organ was homogenized and disrupted using a Polytron as described previously. Serum and urine were examined without PBS-TW dilution. The materials and methods to conduct the ELISA and Western immunoblot are described previously in Chapter 3.

Interpretation of ELISA Assays and Statistical Analysis

Several assumptions made with regards to the interpretation of the ELISA assays, based on data presented in Chapter 3, and on recently published studies by Kokai-Kuhn and McClane (1996). First, that the ELISA assay could detect both free CPE and CPE-R1 receptor bound small complex noting that the small complex moiety had to be in the soluble fraction. Second, that CPE polyclonal antibodies (CPE-pAb) used in the ELISA assay had a higher affinity for free CPE, and thus would preferentially bind to this moiety. This is also stoichiometrically logical since CPE binding to R1 would reduce the number of epitopes available for CPE-CPEpAb binding. Third, low (sublethal) levels of CPE would not saturate R1 receptors in most organs, while high (lethal) levels of CPE would saturate R1 receptors in most

organs. Thus, when administering CPE IP at high levels, unbound CPE would preferentially bind to the CPE-pAb resulting in a positive ELISA ($\alpha = 0.05$). See Chapter 3 for discussion. If the ELISA was positive at $\alpha = 0.2$, CPE may be detected as a mixture of unbound, small complexed and large complexed CPE. Alternately, at sublethal CPE levels (without receptor saturation) CPE detection might be predominantly as a mixture of small complex and large complex forms. Statistical analysis was performed as described in Chapter 3.

Results and Discussion

Symptoms During Toxicosis

Immediately after CPE-A administration the animals presented an accelerated heart rate and a spasmatic breathing pattern (hyperpnea). Animals exhibited arched backs, ruffled-opaque fur, opaque eyes, disorientation, loss of appetite for both food and water, and a requirement for group association in the corners of the cage (gathering). The CPE toxin appeared to express apparent neurotoxicity where animals exhibited flaccid paralysis, suggesting that the vagus nerve had possibly been compromised. At no time did animals present any apparent signs of pain, and until recovery or death the animals would cuddle and rest. When animals recovered from the toxicosis they showed no signs of any long term pathophysiological damage, and ate and drank as before CPE administration. Similar symptoms in animals due to CPE toxicosis were observed by Tsai and Riemann (1975a, 1975b) and Skjelkvale et

al. (1980) although not in as much detail. The toxicosis presented differences in onset time and recovery dependent on the route of administration and the purity of CPE preparations.

Pathological Findings at Necropsy

At necropsy the liver, thymus and kidneys showed petechiae. Some animals had blood in the bladder, while others had an enlarged liver and/or kidneys. At high toxin levels the animals had a mushy bowel with distinct signs of proteolysis of the intestinal walls. The integrity of the blood vessels seemed to be compromised as blood was found in the peritoneal and thoracic cavity. It was extremely difficult to collect blood, and the average blood volume collected was about 1 ml. This was likely due to two factors: first, the animals were very small, and only had a total blood volume of 1.5-3.0 ml. Second, blood coagulation seemed to be accelerated in CPE treated animals compared to control animals. This observation has been confirmed in studies by Wallace et al. (1997). The liver in CPE treated animals was heavier than in controls, suggesting that the toxicosis causes a higher metabolic rate (detoxifying mechanism) which is an attempt to clear CPE from the body. Thus more blood and/or fluids were present in the organ(s), and either upon organ failure or death the fluids were trapped.

Table 4.1 ELISA detection of unbound CPE in murine organ tissues after *in vivo* IP administration.

	CPE dose (MLD*) administered					
Organ	0.1	0.25	0.5	1.0	2.0	5.0
Brain	--	--	--	--	++	++
Thymus	--	--	++	++	++	++
Heart	--	--	--	--	--	--
Lung	--	--	++	++	++	++
Liver	--	--	--	--	--	--
Kidney	--	--	--	--	--	--
Bowel	--	--	--	--	--	++
Blood	--	--	--	++	++	++
Urine	--	--	++	++	++	++

CPE dose administered:
0.1 MLD (0.5 μg/250 μl)
0.25 MLD (1.25 μg/250 μl)
0.5 MLD (2.5 μg/250 μl)
1.0 MLD (5.0 μg/250 μl)
2.0 MLD (10.0 μg/250 μl)
5.0 MLD (50.0 μg/250 μl)
control: 250 μl of PBS

* Mouse Lethal Dose.

(--) = no free CPE detected [$\alpha = 0.05$]
(++) = free CPE detected [$\alpha = 0.05$]

CPE Concentration Study

When CPE was administered at sublethal doses (0.1 and 0.25 MLD) none of the ELISA assays for any of the seven organs examined or blood (as serum) and urine was statistically significant at $\alpha = 0.05$ level (Table 4.1). This suggested two alternatives: first, CPE at this low level was being rapidly metabolized by the liver and kidney, and excreted in the urine before the animals were necropsied and screened by ELISA. Second, the low CPE levels did not saturate cellular receptors as suggested in the section Interpretation of ELISA Assays. Thus neither unbound CPE nor small complex was available for detection. These results are consistent with the pathological findings as none of the animals died within the 72 hour time period after CPE administration. Indeed, while animals presented clear symptoms of CPE induced toxicosis they were able to recover within 8 to 12 hours.

CPE administered at sublethal-lethal levels (0.5 to 1.0 MLD) was detected as free toxin in thymus and lung tissue, and urine ($\alpha = 0.05$). All other organ tissues (brain, heart, liver, kidney, and bowel) were not statistically significant ($\alpha = 0.05$). Serum was found to contain free toxin ($\alpha = 0.05$) at 1.0 MLD but not at 0.5 MLD. Animal sensitivity and resistance to CPE between 0.5-1.0 MLD showed varied responses. Two patterns of sensitivity and resistance were apparent. First, 30-50 % of animals appeared resistant since they effectively recovered from the toxicosis within 8-12 hours of CPE administration. Animals sensitive to CPE died predominantly within the first 24 hours. This bimodal distribution of effects was

consistent through several studies, and was in part not unexpected, since the toxin concentration was near the LD_{50}. Normally within a homogeneous population of mice (same species, similar age and weight) two subgroups are apparent that differ in their ability to metabolize a toxin. The nature or reason for this variability remains unknown, although it is likely that detoxifying mechanism(s) of an individual animal may be less effective and compromised.

All mice administered CPE at 2.0 MLD died within 12 hours, with about 80% of mice dying between 8 and 9 hours. At 5 MLD all mice died within 15 minutes. Free CPE was detected ($\alpha = 0.05$) in brain, thymus, lung and bowel tissues, and blood and urine. No free toxin was apparent in heart, liver and kidney tissues, which was in part unexpected. Several alternatives were suggested: first, none of these organs were saturated with toxin at these levels, and any toxin present in these tissues was present in a form (possibly large complex ?) that was undetectable. Second, due to multiple organ failure, any damaged tissue containing CPE was removed from the organ via the circulation. Third, the animals died from the CPE toxicosis before detectable levels of free toxin were reached. This latter alternative is applicable for CPE at 5 MLD where the toxin likely acted as a neurotoxin.

Table 4.2 Detection of unbound CPE by ELISA in murine organ tissues after *in vivo* IP administration: time study.

	CPE binding time in hours					
Organ	0.25	1.0	2.0	3.0	4.0	5.0
Brain	--	--	--	++	++	++
Thymus	--	--	--	--	--	--
Heart	--	--	--	++	++	++
Lung	--	--	--	--	--	+
Liver	--	--	--	++	++	++
Kidney	--	--	--	--	--	--
Bowel	--	--	--	--	--	--
Blood	++	+	+/-	--	--	--

Treatments: administration of 10 μg/250 μl (2 MLD*) CPE, animals necropsied at 0.25, 1.0, 2.0, 3.0, 4.0, and 5.0 hours; control: 250 μl of PBS.
* Mouse Lethal Dose.

(--) = no free CPE detected [$\alpha = 0.2$]
(+/-) = free CPE detected [$\alpha = 0.2$]
(+) = free CPE detected [$\alpha = 0.1$]
(++) = free CPE detected [$\alpha = 0.05$]

Time Study

Based on data obtained from the concentration study a toxin level of 2 MLD (50 μg/250 μl) was chosen for the time study. This level was chosen for two reasons: first, CPE could be detected with statistical significance and reliability by ELISA, and second, after toxin administration, most mice died within a narrow time-frame 8-9 hours and there was no random death during first 5 hours.

Results from the time study at the single CPE level are shown in Table 4.2. Free toxin was detected in serum within 15 minutes of CPE administration, however, toxin was apparently cleared within three hours. Toxin was detected ($\alpha = 0.05$) in brain, heart, and liver tissues 3 hours after administration, and in the lungs ($\alpha = 0.1$) after 5 hours. CPE was not detected in thymus, kidney or bowel tissue ($\alpha = 0.2$). Since CPE is systemically absorbed and distributed, its presence in brain, heart, liver and lung tissue was not unexpected. However, apparent lack of presence in thymus and kidney was unexpected. Lack of presence in bowel was not unexpected since, this tissue contains a high number of receptors. More importantly however, for toxin to be present in ileal tissue would require translocation of toxin from the peritoneal cavity to the lumen, against the normal translocation gradient. Alternately, lack of free toxin in thymus and kidney may also suggest that in these tissues, that if CPE is present, it is in the large complex form and therefore inaccessible to CPE-pAb. Another consideration can be made. It is possible that toxin distribution and binding does not follow a set distribution pattern. That is distribution and binding may

Table 4.3 ELISA and Western immunoblot detection of CPE (free toxin) CPE:R1 (small complex) and CPE:R1:R2 (large complex) in murine organ tissues after *in vivo* IP administration.

CPE concentration and binding conditions

		Western immunoblot		
Organ	ELISA	35	85	160
Brain	--	--	■	■
Thymus	--	--	■	--
Heart	--	■	■	--
Lung	--	■	--	--
Liver	--	--	--	--
Kidney	--	--	■	■
Bowel	--	--	■	--

CPE dose administered: 0.1 MLD (0.5 μg/250 μl)
control: 250 μl of PBS

ELISA
(--) = no free CPE detected [α = 0.2]

Western immunoblot
35: M_r 35 kDa (free CPE); 85: M_r 85 kDa (CPE:R1);
160: M_r 160 kDa (CPE:R1:R2).
(--) = no band detected
(■) = band detected

depend on how CPE was systemically absorbed, the time needed for toxin to be absorbed, exact location of IP administration, or other factors inherent to the animal itself, for example the metabolism of the individual animal.

It is interesting to compare the results obtained for the concentration and time studies at the same CPE levels. It appears that there are differences in toxin distribution patterns regarding thymus, heart, and liver tissues. CPE was detected in the free form in the time study, but not in the concentration study in heart tissue, while the liver and thymus were negative ($\alpha = 0.1$) . One possible explanation for these differences was that the sample size of the two studies was different. Also, the time allowed for CPE distribution and subsequent binding to receptors was different. In the time study the samples were collected within the first 5 hours, while in the concentration studies samples were collected between 8-12 hours. Since it was not known how time affected CPE distribution and binding, we chose not to directly compare the ELISA data from the time and concentration studies, but to further analyze each separately.

Western Immunoblot

From the *in vitro* studies it was presupposed that CPE bound to various organ tissues within the mouse, albeit with different affinities. It was also assumed that CPE bound initially to receptor R1 forming small complex, and then to R2 forming large complex. The ELISA data predicts the presence of CPE on various organs.

However, in order to confirm CPE presence and determine the conformation and configuration state (small or large complex) of the toxin it was necessary to conduct analysis by Western immunoblot. It should be noted that sample preparation for each method was significantly different. ELISA samples required no special preparation steps, and simply screened the supernatant for soluble protein (antigen). Western's required tissue samples to be boiled in the presence of ß-mercaptoethanol and SDS, allowing the release of tissue associated proteins. In order to initially determine the sensitivity of the Western immunoblot assay, all tissue samples from the 0.1 MLD concentration study were screened for the presence of CPE. These samples were chosen since they represented the lowest level of CPE administration, and the ELISA assay was not able to detect free CPE in any samples. Previous work had indicated that the sensitivity of the ELISA assay in this study was $\geq$ 100 pg CPE/ml, whereas the sensitivity for the Western assay $\geq$ 1 pg CPE/ml. It was assumed that if CPE was present in tissue it had to be found either as the small (85 kDa) or more likely the large (160 kDa) complex. A summary of the analyses is shown in Table 4.3. As previously noted, at 0.1 MLD the ELISA did not detect any unbound toxin as the M_r 35 kDa protein in any tissue. However, Western analysis of CPE treated animals showed a 35 kDa protein band in the heart and lung which was not present in control (untreated animals). Whether toxin was present in these organs as free (unbound) toxin not detectable by ELISA, or as loosely bound small complex which dissociated upon sample preparation is unknown. A 85 kDa band, presumably small complex

was detected in the brain, thymus, heart, kidney and bowel tissues. This band was not present in control (untreated samples). A 160 kDa band presumably large complex was present in brain and kidney tissues, and again this band was not present in control tissues.

The Western analyses suggest that either the ELISA assay was less sensitive to CPE detection when toxin was administered at nonlethal levels or that at nonlethal levels CPE was bound as small and large complex, and thus not available for detection. The latter is more likely since the number of R1 receptors exceeds the number of CPE molecules, and thus receptor saturation was not reached. The Western assay was very informative since it allowed determination of the state and type of CPE association to specific receptors (either small or large complex). Both the ELISA and Western assays compliment each other since the ELISA determines receptor saturation threshold, while the Western determines the type and distribution of CPE-receptor interaction. The type of CPE-receptor complex formed appeared to be specific within individual organs, that is, different organs showed different CPE-receptor complex patterns. These patterns may suggest that toxin distribution within an individual tissue is both CPE-concentration dependent and cell type specific.

The intraperitoneal studies showed that CPE is systemically distributed throughout the body within minutes of administration. The pathophysiological effects of this rapid distribution were both concentration and time dependent. At nonlethal levels CPE was not detected in blood, however, it is likely that at these concentrations

the toxin was rapidly bound to receptors within the liver and kidneys, and metabolized and excreted via urine. The basis for this assumption is first, that both the liver and kidney contain the highest number of CPE-receptors. Second, the receptors within these organs appear to have the highest affinity for CPE (McDonel, 1980). Third, these two organs are normally associated with the removal of toxic products from the body. At sublethal levels CPE appears to have several modes of action. First, as a cytotoxic-enterotoxin causing diarrhea accompanied by gross tissue damage to the bowel. Second, as a neurotoxin since animals presented symptoms usually termed parasympathomimetic (cholinergic). This neurotoxic effect, confirms previous descriptions associated with CPE toxicosis in animals and humans (Murrell et al. 1987; Lindsay, 1996). At lethal levels CPE appears to act as both a neurotoxin and a superantigen. Death is rapid, and symptoms of bradycardia, hyperpenia and flaccid paralysis were apparent, leading to multiple organ failure, shock and death. These symptoms suggest a complete collapse of the sympathetic nervous system accompanied by the massive induction of cytokines at the cellular level. While these findings apply to intraperitoneal administration of CPE it remains to be determined whether similar whole body distribution patterns and responses occur after intragastric administration. This is critical since CPE is normally synthesized, activated and systemically absorbed in the small bowel. This is the focus of Chapter 5.

CHAPTER 5
DETECTION AND DISTRIBUTION OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN AFTER *IN VIVO* INTRAGASTRIC ADMINISTRATION INTO SWISS WEBSTER MICE

Introduction

The effects of *in vivo* oral administration of *C. perfringens* type A vegetative cells, spores suspensions, sterile culture filtrates, and crude cell preparations containing type A enterotoxin (CPE) into various animal models has been studied previously by several groups (Canada and Strong, 1965; Weiss et al., 1965; Hauschild et al., 1971; Uemura et al., 1975; Tsai and Riemann, 1975a, 1975b). Unfortunately, either the basis for the studies were questionable or the results were inconclusive. Canada and Strong (1965) force-fed germ-free mice with live *C. perfringens* type A suspensions, and measured the heat resistance of the spores recovered after passage through the animals GI tract. As would be expected the recovered spores had the same heat resistance as those initially fed the animals. What this study showed or proved is moot. In other studies it appeared that either the administration or absorption levels were too low, thus enterotoxin could not be detected in serum, and the only signs of toxicosis were emesis and diarrhea (Weiss et al., 1965; Hauschild et al., 1971; Uemura et al., 1975).

Tsai and Riemann (1975a, 1975b) examined the responses of mice to oral challenge with *C. perfringens* type A vegetative cells, spore suspensions, and a crude

C. perfringens cell supernatant containing unquantified levels of CPE. Challenge with vegetative cells and spore suspensions showed the bacterium to be present in the gastrointestinal (GI) tract, heart, lung, liver, kidney, spleen, and blood. The presence in various organs is interesting, however, there was no indication that the bacterium was actually bound within tissue. Presence could merely have been a function of systemic distribution via blood, and lack of washing prior to analysis. Unfortunately there was no determination of the level of CPE in any of these tissues or within blood. Administration of crude supernatants containing CPE, and for that matter any other *C. perfringens* toxins, showed CPE present in serum. Mice also exhibited diarrhea, and mucosal enteritis with edema and hyperemia of the small intestine. Whether these pathophysiological responses were due to CPE or any of the other *C. perfringens* toxins was not determined.

The work of Tsai and Riemann (1975a) is perhaps the best of the previous studies, since it did show that CPE can be found in blood after oral administration. However, several criticisms of their work can be made. First, it is impossible to determine the level of toxin absorption let alone the amount of CPE produced in the small intestine simply based on feeding live cultures. There is significant variation in sporulation between *C. perfringens* strains *in vitro*, and the level of sporulation and CPE production *in vivo* is simply unknown, except for the 8239 lab strain. Even for this strain there are many variables that interplay *in vivo*. Second, were there any other *C. perfringens* toxins produced and absorbed, since this bacterium may produce

up to 15 different toxins, many of which appear to act synergistically? Third, the use of crude toxin containing supernatants is fraught with problems, since as noted, many toxins act synergistically. In a crude extract how can any one toxin be shown to cause a specific pathological or histopathological response. Thus pure preparations of a specific toxin have to be used to determine cause and effect. Fourth, the time frame used to study any pathological effects needs to be extended. Distribution studies should at least mimic FBI symptoms which usually take 1-24 hours, or possibly longer to elicit. Further, a range of toxin doses should be administered to determine if there are dose and time dependent responses. Fifth, toxin detection or effect should not be investigated by a single type of assay, since a single assay may provide either or both, false positives and/or false negatives. Thus, several methods of analyses must be used, one of which must be biological. Finally, any method used, has to have the ability to be statistically validated. This chapter reports studies to determine the whole body distribution of CPE toxin after intragastric administration through the natural port of entry, that is intragastrically, using the murine model. The criticisms and requirements discussed above have been incorporated into the study, in order to specifically define the response.

Materials and Methods

CPE-Toxin and Antisera

Freeze dried CPE obtained from Dr. Bruce McClane was prepared for IG administration as described in Chapter 3. The specific activity of preparations was examined before use by Vero cell analysis (Mach and Lindsay, 1994) and was standardized to 4,000 EU/μl toxin. The methods to produce antisera to CPE are described in Chapter 3.

Animal Preparation and IG Enterotoxin Administration

Animals were purchased and maintained as described in Chapter 3. All mice were bred from the same line and obtained from the same source. It was assumed that they were genetically similar, and after gross examination no phenotypic differences were apparent. When animals weighed 15-18 g (25-30 days old), they were randomized and grouped six/cage. To reduce any administration differences, one person (Keller) performed all toxin preparation and oral administration (gavage). Animals were IG administered CPE via a 250 μl volume into the oesphagous and stomach, using a 1 ml tuberculin syringe and a 27 gauge, 2 mm ball-ended Popper needle. Preliminary studies indicated that it was not necessary to neutralize stomach acid using 50 mM bicarbonate buffer prior to CPE administration. Prior administration was observed to upset the mice and vary the biological response after

CPE administration. Additionally, not using bicarbonate buffer more closely mimicked the true toxicosis, since it allowed protease activation of the toxin in the small intestine. CPE retained biological activity in the presence of PBS, and since the volume administered was quite small, delivery was relatively straight-forward. After CPE administration mice were returned to their respective cages and monitored every 15 minutes.

Study 1a: CPE Concentration

This study was performed to determine several factors. First, the CPE concentration that caused death within 72 hours, which would allow a direct comparison between the IP MLD obtained in Chapter 4, and the IG MLD. Second, the CPE concentration at which any pathophysiological changes observed during toxicosis could be strongly predicted without interference from cases of random death within the treated mouse population. This would then allow an IG time concentration study to be conducted similar to that performed in Chapter 4. Thus CPE administration, necropsies and tissue sampling could be done within a specific time frame with reliability.

CPE was administered IG at various levels ranging from 25-75 μg CPE (0.1-0.25 MLD) in 250 μl PBS. Twelve mice were used at each treatment level, and 3 control mice were administered PBS alone. Mice were monitored for pathophysiological changes every 15 minutes from T_o (immediately after

administration) to T_{72} hours, or time to death, which ever came first. Mice were necropsied and all organs (brain, thymus, lung, heart, liver, kidney and small bowel) were isolated, washed in PBS-TW, weighed and transferred to sterile 15 ml polypropylene tubes and stored at -70°C for analysis.

Study 1b: CPE Distribution vs Time

In retrospect the data obtained in Study 1a (see Results and Discussion for details) proved to be of limited value, since toxin concentrations within this range were nonlethal. Rather than conduct additional studies using large numbers of animals to determine the IG MLD, two mice were each administered CPE IG at concentrations of 100, 125, 150, 250 and 500 μg/250 μl and the time to death determined. This two mouse method has been used previously to determine the MLD of *C. botulinum* neurotoxin with good statistical reliability (Sugiyama, 1986). Based on these additional data two CPE concentrations, 150 μg CPE/250 μl PBS (0.5 MLD) and 500 μg CPE/250 μl PBS (2.0 MLD) were chosen for the distribution vs time studies. For each time study, ten mice were administered CPE IG in a single dose, nine similar weighted control mice were administered PBS alone.

Each toxin level was initially designed as a distribution vs time study, however, for reasons discussed in the Results and Discussion section each study presented its own unique problem. Unexpectedly at each of the CPE concentrations, two distinct time to death patterns were apparent. That is, despite the genotypic and

phenotypic similarity of the mouse populations used for each study, half the population died within one time period, the other half within a different time period. Therefore the data were analyzed taking into account the time to death variable. Mice administered CPE were necropsied immediately upon death. At the same time one matched control mouse was euthanized and necropsied. All organs (brain, thymus, lung, heart, liver, kidney, spleen, stomach, and small bowel) were collected and stored as described previously.

Immunological Methods and Statistical Analysis

To prepare organ tissue samples for ELISA, 3 ml of PBS-TW was added, and each organ was homogenized and disrupted using a Polytron as described previously. Serum and urine were not examined. The materials and methods required to conduct the ELISA and Western immunoblot are described previously in Chapter 3. The same assumptions for the interpretation of the ELISA assay and statistical analysis as described in Chapter 4 were used in the IG studies.

Vero Cell Assay

Tissue suspensions of all organs were tested for biologically active CPE using the Vero cell assay (African Green Monkey kidney cells:ATCC CCL81) (Mach and Lindsay, 1994). Cells were cultured in Sarstedt (Newton, NC) 75 cm^2 flasks and maintained in modified Eagles medium (MEM) containing 10% fetal bovine serum

(FBS), 2 mM L-Glutamine, and antibiotic/antimycotic (Sigma Chemical Co.). Cells were incubated as an adherent monolayer in a humidified incubator with 5% CO_2/95% air at 37°C. When confluent, cells were removed with trypsin-EDTA (Sigma Chemical Co.), diluted to 10 ml in MEM and plated 0.2 ml/well (10^5 cells/ml) in Costar 48 well cluster dishes. Cluster dishes were incubated as described above for 24 hours, the media removed and each well washed twice with sterile 15 mM PBS. Earl's balanced salts solution (free of FBS) containing 0.28 mM phenol red (BSS-PR) was added to the wells (final well volume of 0.2 ml). To test the biological activity (cytotoxicity) of any CPE in the high-speed centrifuged tissue supernatant, 100 μl of BSS-PR was replaced with 100 μl of tissue supernatant and incubated for 2 hours. Cells were examined for morphological damage and viability with a Nikon phase contrast microscope. A sample was considered biologically active if it caused $>$ 50% cell lysis in duplicate wells. To determine whether cell death was caused by CPE, all suspected CPE positive tissues as determined by a positive ELISA, positive Western and positive Vero cell assay, were re-assayed in the Vero cell assay using CPE neutralizing antibody. Tissue supernatants were treated with a 1:100 dilution of CPE primary antibody, thus neutralizing any available toxin. Any sample which was ELISA, Western and Vero cell assay positive, and negative in the Vero cell assay after neutralization with anti-CPE antibody, was considered a confirmed positive.

Results and Discussion

Symptoms During Toxicosis

To induce a toxicosis by IG administration required a 10-15 fold higher CPE level. Upon IG CPE administration, mice presented similar symptoms and pathophysiological changes as observed for IP CPE administration, see Chapter 4 for detailed description. Mice administered nonlethal CPE levels fully recovered from the toxicosis within 6-8 hours. Animals administered sublethal levels of CPE, presented two response modes. Approximately 25% of the mice died within 4 hours, while the remaining 75% recovered within 6-8 hours. Although the animals used were genetically and phenotypically similar, this response was not unexpected since the amount of toxin administered was near the 0.5 MLD. Administration of lethal levels of CPE caused the death of all animals, however, two modes of death were apparent. First, a "sudden" death induced within minutes of toxin administration, and second, a nonabrupt death that required several hours for manifestation.

Pathological Findings at Necropsy

At nonlethal CPE levels there was no apparent gross histopathological tissue damage to organs, other than the bowel. At sublethal and lethal CPE levels the liver, thymus, and kidneys showed petechiae, and the liver and kidneys were enlarged. Enlarged organs suggest hyperactivity which leads to organ failure. Additionally, the GI tract exhibited signs of extensive proteolysis (mushy and spongy) to the

submucosa, and without care upon dissection the tissue would disintegrate. It was apparent that the damage to the digestive tract would significantly compromise metabolism to the point where animals had very little chance of recovery. This type of bowel tissue damage has been observed for other *C. perfringens* infections, for example pigbel, where recovery from the disease requires surgery. Collection of blood was not possible since the blood vessels in the peritoneal cavity showed signs of hemorrhage. Coagulated blood was found in the peritoneal cavity, lungs, liver, kidneys and digestive tract. This may have contributed to a decrease in blood volume within the animal. Wallace et al. (1997) observed similar responses in their work, and preliminary data indicated that the coagulation factors appeared to be affected during CPE-toxicosis. No urine could be collected during necropsy likely due to kidney organ failure, or possibly loss of involuntary control of the urinary system.

CPE Concentration Study

The ELISA assay did not detect free CPE administered at 0.1 MLD (nonlethal) in any organ-tissue sample (Table 5.1). This was expected since nonlethal levels would not have saturated tissue receptors, and thus toxin was not available for detection. At 0.25 MLD the brain and kidney had free CPE at a significance level of $\alpha = 0.2$, but all the other organs showed no free CPE (Table 5.1). This may suggest that at 0.25 MLD the CPE threshold for receptor saturation in brain and kidney tissues was reached. However, since the kidney is the main organ of CPE

Table 5.1 Detection of unbound CPE by ELISA in murine organ tissues after *in vivo* IG administration.

	CPE dose administered	
Organ	0.1 MLD	0.25 MLD
Brain	--	+/-
Thymus	--	--
Heart	--	--
Lung	--	--
Liver	--	--
Kidney	--	+/-
Bowel	--	--

CPE dose administered: 0.1 MLD (25 μg/250 μl)
0.25 MLD (75 μg/250 μl)
controls 250 μl of PBS

Statistics
(--) = no free CPE detected [$\alpha = 0.2$]
(+/-) = free CPE detected [$\alpha = 0.2$]

Table 5.2 ELISA and Western immunoblot of CPE (free toxin) CPE:R1 (small complex) and CPE:R1:R2 (large complex) in murine organ tissues after IG administration.

	CPE concentration and binding condition			
	0.25 MLD			
Organs	ELISA	Western		
		35	85	160
Brain	+/-	--	■	■
Thymus	--	--	■	■
Heart	--	--	■	■
Lung	--	--	--	■
Liver	--	--	--	■
Kidney	+/-	--	■	■
Bowel	--	--	■	■

CPE binding conditions: administration of 0.25 MLD (75 μg/250 μl) controls were administered 250 μl of PBS

Statistics: (--) no free CPE detected [$\alpha = 0.2$]
(+/-) free CPE detected [$\alpha = 0.2$]

Western: 35: M_r 35 kDa (free CPE); 85: M_r 85 kDa (CPE:R1)
160: M_r 160 kDa (CPE:R1:R2); (■) band; (--) no band

detoxification and excretion, free CPE might be expected. Whether the CPE-receptors in brain tissue were saturated is unknown, however, based on the *in vitro* studies this would appear likely.

Western immunoblots of tissues from animals administered 0.25 MLD showed CPE not as free CPE, but rather as a combination of small (CPE-R1) and large (CPE-R1-R2) complex (Table 5.2). These results confirm Kokai-Kun and McClane's (1996) studies showing that the ELISA assay could detect CPE when it is exposed but sequestered in small or large complex. The statistical significance of the ELISA results are lower than perhaps expected ($\alpha = 0.2$) however, the binding coefficient of free CPE to the primary antibody is higher than bound toxin. Thus, as noted previously in Chapters 3 and 4, the sensitivity of the ELISA is lower when it is detecting CPE as a mixture of complexes and free toxin. An interesting and as yet unexplained finding was that lung and liver tissues contained CPE predominantly as large complex.

Sensitivity differences between the ELISA assay and the Western immunoblot have been discussed previously in Chapter 4. However, it is important to stress that while the assays are different, that is, the immunoblot specifically discerns between free CPE and CPE-complexed forms, the ELISA predominantly detects free CPE in tissue supernatants (due to a higher binding coefficient). Thus the methods become complimentary.

Time Study

Since there was negligible tissue damage upon administration of nonlethal amounts of CPE no time studies were conducted at this level. ELISA results of administration of sublethal CPE levels (0.5 MLD) showed free toxin (significant at $\alpha = 0.05$ level) in thymus, heart, and bowel tissues, and (significant at $\alpha = 0.1$ level) in kidneys (Table 5.3). Brain, lung, liver, stomach and spleen as assayed by ELISA contained no free CPE. As discussed previously two patterns of responses were found at sublethal levels, recovery or death. Comparison of CPE time-distribution patterns between these two groups showed remarkable similarities except for the kidneys. Animals that died within 4 hours after toxin administration had free CPE, while kidneys of animals that recovered from the toxicosis had no significant free CPE. This is not unexpected since animals that recovered from the toxicosis obviously metabolized and excreted toxin via urine.

Western immunoblots confirmed the presence of free CPE in all organs except in bowel and spleen tissues (Table 5.4). Brain, thymus, heart, lung, liver, and kidney tissues also had CPE in the small and large complex form. The stomach tissue contained only free CPE strongly suggesting that this organ has no CPE receptors. Free CPE in stomach tissue confirmed that intragastric administration was successful, and that the presence of CPE in lung tissue was the result of systemic distribution not faulty administration. The bowel had no CPE in any form (free or complexes) as detected by Western immunoblot. Possibly, the gross destruction of the small bowel

Table 5.3 ELISA detection of unbound CPE in murine organ tissues after *in vivo* IG administration: time study.

	CPE binding time in hours	
Organ	4 hr.	72 hr.
Brain	--	--
Thymus	++	++
Heart	++	++
Lung	--	--
Liver	--	--
Kidney	+	--
Bowel	++	++
Stomach	--	--
Spleen	--	--

CPE binding conditions: administration of 0.5 MLD (150 μg/250 μl)
animals necropsied either at 4 or 72 hours
controls were administered 250 μl of PBS

Statistics: (--) = no free CPE detected [$\alpha = 0.1$]
(+) = free CPE detected [$\alpha = 0.1$]
(++) = free CPE detected [$\alpha = 0.05$]

Table 5.4 ELISA and Western immunoblot of CPE (free toxin) CPE:R1 (small complex) and CPE:R1:R2 (large complex) in murine organ tissues after IG administration.

	CPE concentration and binding condition			
	0.5 MLD			
Organs	ELISA	Western		
		35	85	160
Brain	--	■	■	■
Thymus	++	■	■	■
Heart	++	■	■	■
Lung	--	■	■	■
Liver	--	■	■	■
Kidney	+	■	■	■
Bowel	++	--	--	--
Stomach	--	■	--	--
Spleen	--	--	--	--

CPE binding conditions: administration of 0.5 MLD (150 μg/250 μl)
animals necropsied at either 4 and 72 hours
controls were administered 250 μl of PBS

Statistics: (--) no free CPE detected [$\alpha = 0.2$]
(+) free CPE detected [$\alpha = 0.1$]
(++) free CPE detected [$\alpha = 0.05$]

Western: 35: M_r 35 kDa (free CPE); 85: M_r 85 kDa (CPE:R1); 160: M_r 160 kDa (CPE:R1:R2); (■) band; (--) no band

resulted in the complete elimination of any toxin from this tissue. Desquamated tissue was simply either excreted or proteolytically digested before necropsy.

Lethal CPE administration of 2 MLD also showed a bimodal time response where approximately 50 % of the animals died within the 15 minutes, referred to as "sudden" death, while the remaining 50% died a nonabrupt death requiring several hours for lethal outcome. ELISA results showed free CPE in thymus, liver, kidney, stomach, and bowel tissue ($\alpha = 0.05$), and at $\alpha = 0.1$ heart tissue (Table 5.5). Brain, lung, and spleen tissue had no detectable levels of free CPE. Higher free CPE levels were expected in liver and kidney tissues since these are the organs of detoxification and excretion. Another hypothesis could be proposed that, immediately after absorption in the small intestine CPE reaches the liver and kidney via portal vein compromising these organs. This in turn causes multiple organ failure, shock, and death. Liver and kidney failure could reduce blood pressure and blood flow to other organs. As blood is entrapped in failing organs less CPE reaches other tissues, thus not saturating their receptors. Detection of higher levels of free CPE in more organs than previously observed for lower toxin levels could simply be a function of toxin concentration. Simply stated, as more CPE becomes available a greater number of organ-tissues would reach their CPE receptor-saturation threshold. Western immunoblot results showed CPE both as free toxin, small and large complex in all organs except the stomach and spleen (Figures 5.1, 5.2 and Table 5.6).

Table 5.5 ELISA detection of unbound CPE in murine organ tissues after *in vivo* IG administration: time study.

Organ	Sudden	Nonabrupt
Brain	--	--
Thymus	++	++
Heart	+/-	--
Lung	--	--
Liver	++	++
Kidney	++	++
Bowel	--	++
Stomach	++	++
Spleen	--	--

CPE binding conditions: administration of 2.0 MLD (500 μg/250 μl)
controls were administered 250 μl of PBS
Sudden death: death within 15 minutes
Nonabrupt death: several hours for lethal outcome

Statistics: (--) = no free CPE detected [$\alpha = 0.2$]
(+/-) = free CPE detected [$\alpha = 0.2$]
(++) = free CPE detected [$\alpha = 0.05$]

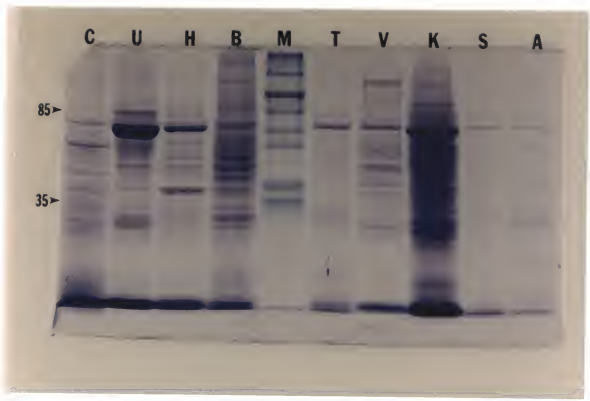


Figure 5.1 SDS-PAGE of tissue supernatants: tissue protein analysis of mice administered 2 MLD IG of CPE. Tissue supernatants from: (C) control tissue homogenate (no CPE), (U) lung, (H) heart, (B) brain, (M) high molecular weight standard, (T) thymus, (V) liver, (K) kidney, (S) spleen, (A) CPE.

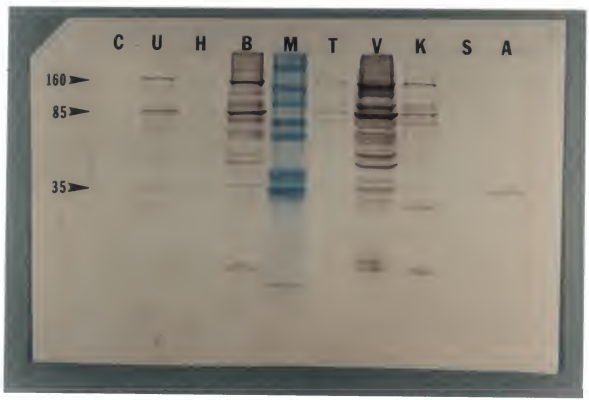


Figure 5.2 Western immunoblot of tissue supernatants: mice administered 2 MLD IG of CPE. Toxin detected with polyclonal antisera to CPE. Tissue supernatants from: (C) control tissue homogenate (no CPE), (U) lung, (H) heart, (B) brain, (M) high molecular weight standard, (T) thymus, (V) liver, (K) kidney, (S) spleen, (A) purified CPE.

Table 5.6 ELISA, Western immunoblot and Vero cell detection of: CPE (free toxin) CPE:R1 (small complex) and CPE:R1:R2 (large complex) in murine organ tissues after *in vivo* IG administration.

	CPE concentration and binding conditions for 2MLD				
Organs	ELISA	Western			Cell death
		35	85	160	
Brain	--	■	■	■	■
Thymus	++	■	■	■	■
Heart	+/-	■	■	■	■
Lung	--	■	■	■	■
Liver	++	■	■	■	0
Kidney	++	■	■	■	0
Bowel	++	■	■	■	■
Stomach	++	■	--	--	■
Spleen	--	--	--	--	0

CPE binding conditions: administration of 2.0 MLD (500 μg/250 μl)
controls were administered 250 μl of PBS

Statistics: (--) no free CPE detected [$\alpha = 0.2$]
(+/-) free CPE detected [$\alpha = 0.2$]
(++) free CPE detected [$\alpha = 0.05$]

Western: 35: M_r 35 kDa (free CPE); 85: M_r 85 kDa (CPE:R1);
160: M_r 160 kDa (CPE:R1:R2); (■) band; (--) no band

Cell death: (■) in Vero cell assay, sample supernatant caused > 50% cell lysis and cell lysis was completely inhibited by neutralization with primary antibody to CPE. (0) no cell lysis

Figure 5.3 Vero cell assay of murine tissues: (A) liver, (B) brain, and (C) bowel tissue supernatants. Animals were administered 2 MLD IG of CPE.

The stomach had only free CPE confirming the ELISA results, and the spleen had no detectable CPE in any form (free or complexed).

The Vero cell analyses concurred with both the ELISA and Western immunoblot results. Free and biologically active CPE was detected in thymus, heart, bowel, and stomach tissue (Table 5.6 and Figure 5.3). Brain, and lung tissue had free biologically active CPE, which confirmed the Western results. The Vero cell assay did not detect free biologically active CPE in liver, kidney or spleen tissues, and these negative results were consistent and reproducible for all samples tested. It would appear that unlike other organ tissues except the stomach, the spleen has no receptors for CPE.

In summary, these findings confirmed the need to use all three assays (ELISA, Western and Vero cell) to elucidate the state of the CPE molecule within any tissue. Although free CPE (35 kDa) appeared to be present within a specific tissue based on the ELISA and Western assays, it did not necessarily mean that the toxin was biologically active. Indeed, free toxin was detected in liver and kidney tissues by ELISA and Western but neither of these tissues contained biologically active CPE.

CHAPTER 6
CONCLUSIONS AND SUMMARY

Clostridium perfringens type A enterotoxin is a known causal agent of foodborne illness in man, and has also recently been implicated as a trigger in the sudden infant death syndrome (SIDS). The pathophysiological changes associated with either disease state appear very complex. Diarrhea and histopathological tissue damage to many organs within the body are a function of the cytotoxigenic and enterotoxigenic nature of the CPE molecule. However, other symptoms observed during the toxicosis suggest both neurotoxigenic (cholinergic) and superantigenic activities. The goal of this research was to better understand the nature of these CPE induced pathophysiological disturbances, by determining where CPE was specifically distributed during toxicosis. The murine model was chosen for this study since as a model it offered many unique genotypic and phenotypic advantages.

In vitro tissue studies showed CPE binding to cellular membrane protein receptors in brain, thymus, heart, lung, liver, kidney, and bowel. Liver, kidney and bowel tissues contained the highest number of CPE-receptors.

Intraperitoneal CPE administration revealed that toxin was systemically and rapidly absorbed, being distributed throughout the body within minutes of administration. CPE induced pathophysiological effects were both concentration and time dependent. All organ tissues within the body except the spleen and stomach

bound CPE, with the liver and kidneys binding the highest amount of toxin. Nonlethal levels of CPE were metabolized by liver and kidneys and excreted via urine. Sublethal CPE levels were both cytotoxic and enterotoxic, causing diarrhea and presenting symptoms associated with neurotoxicity. Lethal CPE levels acted as a neurotoxin and apparently as a superantigen, causing multiple organ failure, shock and rapid death.

Intragastric administration of CPE induced responses that paralleled the intraperitoneal administration findings, except that higher levels of toxin were required to induce similar responses. Results showed that CPE again had three different modes of distribution, but four types of activity which were time and concentration dependent. Nonlethal CPE levels induced enterotoxigenic symptoms, and toxin was metabolized and cleared from the body via urine. Sublethal levels of CPE again induced enterotoxigenic, cytotoxigenic and neurotoxigenic symptoms. Lethal levels of CPE however, induced two patterns of death. First, a "sudden" death induced within minutes of toxin administration, and second, a nonabrupt death that required several hours for manifestation. Animals expressed symptoms of respiratory distress, shock and multiple organ failure, similar to the action of a neurotoxic-superantigen. Based on the various distribution, time and lethality patterns observed in these studies, the following models were developed (Figures 6.1a-d).

Figure 6.1 Murine model for CPE distribution after IG administration

(a) Nonlethal murine model. Animals recover from sequelae within 6-8 hours after IG CPE administration. (+) organs/tissues containing CPE.

(b) Sublethal murine model. Animals may either recover from sequelae or die within 72 hours after IG CPE administration. (+) organs/tissues containing CPE.

CPE Distribution Model: CPE Threshold Saturation Level

- Nonlethal: 0.1 - 0.5 MLD
  - Animals recover from sequelae.

CPE Distribution Model: CPE Threshold Saturation Level

- Sublethal: 0.5- 1.0 MLD
  - Animals may recover from sequelae or die within 72 hours.

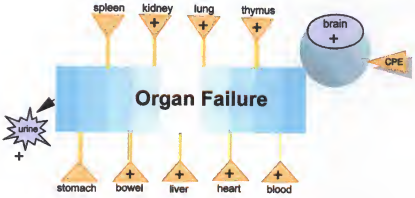


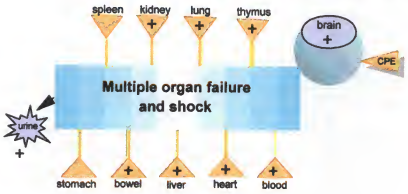
Figure 6.1 continued.

(c) Sudden death murine model. Animal dies within 15 minutes after IG CPE administration. (+) organs/tissues containing CPE.

(d) Nonabrupt death murine model. Animal dies hours after IG CPE administration. (+) organs/tissues containing CPE.

CPE Distribution Model: CPE Threshold Saturation Level

- Lethal dose: > 2.0 MLD
  - Immediate outcome: shock and death within minutes.



CPE Distribution Model: CPE Threshold Saturation Level

- Lethal dose: > 2.0 MLD
  - Nonabrupt outcome: multiple organ failure, shock and death requiring several hours for manifestation.

The murine models may be extrapolated to the human response to CPE toxicosis. Nonlethal levels of CPE may cause a toxicosis similar to a self-limiting foodborne illness. Systemic absorption of sublethal levels of CPE may induce a neurotoxicosis, from which a healthy individual would likely recover, however, death could occur with this toxin concentration in an immunocompromised person, the young or elderly.

Systemic absorption of lethal CPE levels will cause death in all persons. The observation of a "sudden" pattern of death after lethal ingestion was remarkable, and pivotal. This pattern mimics the suggested response of some at-risk infants to a cytotoxic-superantigen toxicosis, and supports a role for CPE as a trigger in some cases of SIDS.

The studies outlined in this dissertation suggest that CPE also induces pathophysiological responses associated with neurotoxicity and superantigenicity. To confirm this hypothesis, future work should be focussed on determining whether cytokines are induced during CPE toxicosis. Specifically, the induction of interferon-gamma, tumor necrosis factor-alpha, and interleukins IL-1, IL-2 and IL-6. Studies should also focus on which cytokines are induced in relation to specific tissues, whether induction is localized, and if the cytokine induction is CPE concentration and time dependent. Additionally, if CPE has a role as a trigger for death in SIDS which mimics the "sudden" death murine model (Figure 6.1c) then, increased cytokine levels may be apparent in infants who have died from SIDS.

REFERENCES

Arnon SS (1983) Breast-feeding and toxigenic intestinal infections: missing links in SIDS. In: Tildon JT, Roeder LM, Steinschneider A (eds) Sudden Infant Death Syndrome. New York, Academic Press, pp 539-555

Baron S, Fleishmann WR, Coppenhaver DH, Nielsen DW, Klimpel GR, Stanton GJ, Hughes TK (1991) The interferons: mechanisms of action and clinical applications. JAMA 266:1375-1383

Bartholomew BA, Stringer MF, Watson GN, Gilbert RJ (1985) Development and application of an enzyme-linked immunosorbent assay for *Clostridium perfringens* type A enterotoxin. J Clin Pathol 38:222-228

Bartoszcze M, Nowakowska M, Roszkowski J, Martas J, Palec S, Wystup (1990) Chinchilla deaths due to *Clostridium perfringens* A enterotoxin. Vet Rec 126:342-343

Bean NH, Griffin PM (1990) Foodborne disease outbreaks in the United States, 1973-1987; pathogens, vehicles and trends. J Food Protect 53:804-817

Beckwith JB (1988) Intrathoractic petechial hemorrhages: a clue to the mechanism of death in sudden infant death syndrome? Ann NY Acad Sci 533:37-47

Bettelheim LA, Golkwater PN, Dwyer BW, Bourne AJ, Smith DL (1990) Toxigenic *Escherichia coli* associated with sudden infant death syndrome. Scand J Infect Dis 22:467-476

Birkhead G, Vogt RL, Heun EM, Snyder JT, McClane BA (1988) Characterization of an outbreak of *Clostridium perfringens* food poisoning by quantitative fecal culture and fecal enterotoxin measurement, J Clin Microbiol 26:471-474

Blackwell CC, Weir DM, Busuttil A, Saadi AT, Essery DE, Raza MW, James VS, Mackenzie DA (1994) The role of infectious agents in sudden infant death syndrome. FEMS Immunol Med Microbiol 9:91-100

Blackwell CC, Weir DM, Busuttil A (1995) Infectious agents, the inflammatory responses of infants and sudden infant death syndrome (SIDS). Mol Med Today 1:72-78

Bouvier-Colle MH, Flahaut A, Messiah A, Jougla E, Hatton F (1989) Sudden infant death and immunization: an extensive epidemiological approach to the problem in France-winter 1986. Int J Epidemiol 18:121-126

Bowness P, Moss PAH, Tranter H, Bell JI, McMichael AJ (1992) *Clostridium perfringens* enterotoxin is a superatigen reactive with human T cell receptors Vβ6.9 and Vβ22. J Exp Med 176:893-896

Bruce K, Becker L (1992) Quantitation of medullary astrogliosis in SIDS. Pediatr Neurosurg 17:74-79

Brynestad S, Iwanejko LA, Stewart GSAB, Granum PE (1994) A complex array of Hpr consensus DNA recognition sequences proximal to the enterotoxin gene in *Clostridium perfringens* type A. Microbiol 140:97-104

Buck GM, Cookfair DL, Michalek AM, Nasca PC, Standfast SJ, Sever LE, Kramer AA (1988) Intrauterine growth retardation and risk of sudden infant death syndrome. Am J Epidemiol 129:874-884

Canada JC, Dorothy HS (1965) Effects of animal alimentary passage on the heat resistance of *Clostridium perfringens*. Appl Microbiol 13:788-792

Canard B, Saint-Joanis B, Cole ST (1992) Genomic diversity and organization of virulence genes in the pathogenic anaerobe *Clostridium perfringens*. Molec Microbiol 6:1421-1429

Centers for Disease Control (1994) *Clostridium perfringens* gastroenteritis associated with corned beef served at St. Patrick's Day meals; Ohio and Virginia 1993. Morbidity and Mortality Weekly Reports 43:137-144

Cerami A (1992) Inflammatory cytokines. Clin Immunol Immunopath 62:S2-S10

Chonmaitree T, Baron S (1991) Bacteria and viruses induced production of interferon in the cerebrospinal fluid of children with acute meningitis: a study of 57 cases and the review. Rev Infect Dis 13:1061-1065

Collier RJ, Kaplan DA (1985) Immunotoxins. Sci Amer 9:56-64

Cornillot E, Saint-Joanis B, Daube G, Datayama S, Granum PE, Canard B, Cole ST (1995) The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Molec Microbiol 15:639-647

Crowther JR (1995) ELISA: theory and practice. Tontowa, NJ, Humana Press

Czeczulin JR, Collie RC, McClane BA (1996) Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B and C isolates of *Clostridium perfringens*. Infect Immunol 64:3301-3309

Czeczulin JR, Hanna PC, McClane BA (1993) Cloning, nucleotide sequencing and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. Infect Immun 61:3429-3439

Daube G, Simmon P, and Kaeckeenbeeck A (1993) IS1151, an IS-like element of *Clostridium perfringens*. Nucleic Acids Res 21:352

Duncan CL, Strong DH (1968) Improved medium for sporulation of *Clostridium perfringens*. Appl Microbiol 16:82-89

Duncan CL, Strong DH (1969) Ileal loop fluid accumulation and production of diarrhea in rabits by cell-free products of *Clostridium perfringens*. J Bacteriol 100:86-94

Duncan CL, Strong DH, Sebald M (1972) Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. J Bacteriol 110:378-391

Fekety R, Kim K, Gatts DH, Browne RA, Cudmore MA, Silva J, Toshniwal R, Wilson K (1980) Studies on the epidemiology of antibiotic-associated *Clostridium difficile* colitis. Am J Clin Nutr 33:2527-2532

Fleischer B (1994) Superantigens. APMIS 102:3-12

Gaskill ME, Khan SA (1986) Regulation of the enterotoxin B gene in *Staphylococcus aureus*. J Biol Chem 263:6276-6280

Gillan JE, Curran C, O'Reilly E, Cahalane SF, Unwin AR (1989) Abnormal patterns of pulmonary neuroendocrine cells in victims of sudden infant death syndrome. Pediatrics 84:828-834

Granum PE, Harbitz O (1985) A circular dichroism study of the enterotoxin from *Clostridium perfringens* type A. J Food Biochem 9:137-146

Granum PE, Richardson M (1991) Chymotrypsin treatment increases the activity of *Clostridium perfringens enterotoxin*. Toxicon 29:896-900

Granum PE, Shitaker JR, Skjelkvale R (1981) Trypsin activation of enterotoxin from *Clostridium perfringens* type A. Biochim Biophys Acta 668:325-332

Granum PE, Stewart G (1992) Molecular biology of *Clostridium perfringens* enterotoxin.In: Sebald M (ed) Genetics and Molecular Biology of Anaerobic Bacteria. New York, Springler-Verlag, pp 235-247

Granum PE, Whitaker JR (1980) Improved method for the purification of enterotoxin *Clostridium perfringens* type A. Appl Environment Microbiol 39:1120-1122

Granum PE, Whitaker JR, Skjelkvale R.(1980) Trypsin activation of enterotoxin from *Clostridium perfringens* type A. Biochem Biophys Acta 668:325-332

Grether JK, Schulman J (1989) Sudden infant death syndrome and birthweight. J Pediatr 114:561-567

Guilian GG, Gilbert EF, Moss RL (1987) Elevated fetal hemoglobin levels in sudden infant death syndrome. New Engl J Med 316:1122-1126

Guntheroth WG (1989) Theories of cardiovascular causes in sudden infant death syndrome. JACC 14:443-447

Hanna PC, McClane BA (1991) A recombinant C-terminal toxin fragment provides evidence that membrane insertion is important for *Clostridium perfringens* enterotoxin cytotoxicity. Molec Microbiol 5:225-230

Hanna PC, Mietzner TA, Schoolnik GK, McClane Ba (1991) Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. J Biol Chem 266:11037-11043

Hanna PC, Wnek AP, McClane BA (1989) Molecular cloning of the 3'half of the *Clostridium perfringens* enterotoxin gene and demonstration that this region encodes receptor-binding activity. J Bacteriol 171:6815-6820

Hanna PC, Wiekowski EU, Mietzner TA, McClane BA (1992) Mapping functional regions of *Clostridium perfringens* type A enterotoxin. Infect Immun 60:2110-2114

Hauschild AH, Niilo L, Dorward WJ (1971) The role of enterotoxin in *Clostridium perfringens* type A enteritis. Can J Microbiol 17:987-991

Hauser D, Eklund MW, Boquet P, Popoff MR (1994) Organization of the botulinum neurotoxin C1 gene and its associated non-toxin protein genes in *Clostridium botulinum* C468. Mol Gen Genet 243:631-640

Hobbs BC (1979) *Clostridium perfringens* gastroenteritis. In: Riemann H and Bryan FL (eds) Food Borne Infections and Intoxications. New York, Academic Press, pp 131-167

Hobbs BC, Smith SF, Oakley CT, Warrack GH, Cruickshank JF (1953) *Clostridium welchii* food poisoning. J Hyg 51:74-101

Hoffman JH, Damus K, Hillman L, Krongrad P (1988) Risk factors for SIDS: results of the National Institute of Child Health an Human Development Cooperative Epidemiological Study of Sudden Infant Death Syndrome. Arch N Y Acad Sci 533:13-30

Hoffman JH, Jehy C, Damus K, Pakter J, Peterson D, van Belle G, Hasselmayer E (1987) Diphtheria-tetanus-pertussis immunization and sudden infant death: results of the National Institute of Child Health and Human Development Cooperative Epidemiological Study of Sudden Infant Death Syndrome. Pediatrics 79:598-611

Hollander N (1988) Beta-endorphin in the brainstem, pituitary, and spinal fluid of infants at autopsy: relation to sudden infant death syndrome. Forensic Sci Int 38:67-74

Horiguchi Y, Akai T, Sakaguchi G (1987) Isolation and function of a *Clostridium perfringens* enterotoxin fragment. Infect Immun 55:2912-2915

Howatson AG (1992) Viral infection and α interferon in SIDS. J Clin Pathol 45:25-28

Hulkower KI, Wnek AP, McClane BA (1989) Evidence that alterations in small molecule permeability are involved in the *Clostridium perfringens* type-A enterotoxin-induced inhibition of macromolecular synthesis in Vero cells. J Cell Physiol 140:498-504

Ijzermans JNM, Maquet RL (1989) Interferon-gamma: a review. Immunobiol 179:456-473

Jakeman K, Rushton D, Smith H, Sweet C (1991) Exacerbation of bacterial toxicity to infant ferrets by influenza virus: possible role in SIDS. J Infect Dis 163:35-40

Janssen MT, Put HM, Nout MJ (1996) Natural toxins. In: de Vries J (ed) Food Safety and Toxicity. Boca Raton, FL, CRC Press, pp 23-24

Johnson HM, Russell JK, Ponzer CH (1992) Superantigens in human disease. Sci Am 4:92-101

Jones AM, Weston JT (1976) The examination of the sudden infant death syndrome infant: investigative and autopsy protocols. J Forensic Sci 21:833-841

Jones CL, Khan SA (1986) Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. J Bacteriol 162:29-33

Kariks J (1989) Diaphragmatic muscle fibre necrosis in SIDS. Forensic Sci Int 43:281-291

Katayama S, Dupuy B, Daube G, China B, Cole ST (1996) Genome mapping of *Clostridium perfringens* strains with I-*Ceu*I shows many virulence genes to be plasmid borne. Mol Gen Genet 251:720-726

Klein E (1895) Ueber einen pathogenen anaeroben Darmbacillus, *Bacillus enteritidis sporogenes*. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg 18:737

Knox R, McDonald EK (1943) Outbreaks of food poisoning in certain Leicester institutions. Med Officer 69:21-22

Kokai-Kun JF, Songer JG, Czeczulin JR, Chen F, McClane BA (1994) Comparison of western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. J Clin Microbiol 32:2533-2539

Kokai-Kun JF, McClane BA (1996) Evidence that a region(s) of the *Clostridium perfringens* enterotoxin molecule remains exposed on the external surface of the mammalian plasma membrane when the toxin is sequestered in small or large complexes. Infect Immun 64:1020-1025

Krous HF (1984) The microscopic distribution of intrathoracic petechiae in sudden infant death syndrome. Arch Pathol Lab Med 108:77-79

Labbe RG (1989) *Clostridium perfringens*. In: Doyle MP (ed) Foodborne Bacterial Pathogens. New York, Marcel Dekker, Inc., pp 192-234

Labbe RG, Duncan CL (1977) Evidence for stable messenger ribonucleic acid during sporulation and enterotoxin synthesis by *Clostridium perfringens* type A. J Bacteriol 129:843-849

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680-685

Lindsay JA (1988) The effect of a *Clostridium perfringens* 8-6 enterotoxin on viability and macromolecular synthesis in Vero cells. Biochem Biophys Res Comm 151:1371-1377

Lindsay JA (1996) *Clostridium perfringens* type A enterotoxin (CPE): more than just explosive diarrhea. Crit Rev Microbiol 22 (4):257-277

Lindsay JA, Dennison JD (1986a) Histopathological effect of *Clostridium perfringens* 8-6 enterotoxin on rabbit intestine. Curr Microbiol 13:61-66

Lindsay JA, Dennison JD (1986b) A scanning electron microscope study of the effect of an enterotoxin from *Clostridium perfringens* 8-6 on mice of different ages. J Gen Microbiol 132:2839-2898

Lindsay JA, Johnson H, Wallace FM, Soos J (1994) Can superatingens trigger sudden infant death? Med Hypoth 43:81-85

Lindsay JA, Mach A, Wilkinson M, Martin L, Wallace L, Keller A, Wojciechowski L (1992) A role for *Clostridium perfringens* and its enterotoxin(s) in SIDS. A microbiological, toxicological and histopathological study. 2nd International SIDS Conference, Sydney, Australia, 65a, 120

Lindsay JA, Mach A, Wilkinson M, Martin L, Wallace L, Keller A, Wojciechowski L (1993) Clostridium perfringens type A cytotoxic-enterotoxin(s) as triggers for death in Sudden Infant Death Syndrome: Development of a toxico-infection hypothesis. Curr Microbiol 27:51-59

Lindsay JA, Sleigh RW, Ghitgas C, Davenport JB (1985) Purification and properties of an enterotoxin from a coatless spore mutant of *Clostridium perfringens* type A. Eur J Biochem 149:287-293

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265-275

Mach AS, Lindsay JA (1994) Activation of *Clostridium perfringens* cytotoxic enterotoxins *in vivo* and *in vitro*: role in triggers for SIDS. Curr Microbiol 28:261-267

Mach AS, Lindsay JA (1997) *Clostridium perfringens* enterotoxin stimulates *in vitro* nitric oxide production in the presence of cytokines. 97th ASM General Meeting, Miami Beach, Florida, Abstract P-025, pp 441

McClane BA (1992) *Clostridium perfringens* enterotoxin: structure, action and detection. J Food Safety 12:237-252

McClane BA (1994) *Clostridium perfringens* enterotoxin acts by producing small molecule permeability alterations in plasma membranes. Toxicology 87:43-67

McClane BA (1997) *Clostridium perfringens*. In: Doyle M, Montville T, Beuchart L (eds) Food Safety. Washington, DC, ASM Press, pp 305-326

McClane BA, Hanna PC, Wnek A (1988a) *Clostridium perfringens* type A enterotoxin. Microb Pathog 4:317-323

McClane BA, McDonel JL (1979) The effects of *Clostridium perfringens* enterotoxin on morphology, viability and macromolecular synthesis in Vero cells. J Cell Physiol 99:191-200

McClane BA, Wnek AP (1990) Studies of *Clostridium perfringens* enterotoxin action at different temperatures demonstrate a correlation between complex formation and cytotoxicity. Infect Immun 58:3109-3115

McClane BA, Wnek AP, Hulkower KI, Hanna PC (1988b) Divalent cation involvement in the action of *Clostridium perfringens* type A enterotoxin. J Biol Chem 263:2423-2435

McClane B, Wnek A, Whitaker-Dowling P (1987) Interferon pretreatment enhances the sensitivity of Vero cells to *Clostridium perfringens* type A enterotoxin. Microbial Pathog 3:195-206

McClung LS (1945) Human food poisoning due to growth of *Clostridium perfringens* (*C. welchii*) in freshly cooked chickens. J Bacteriol 50:229-231

McDonel JL (1980) Binding of *Clostridium perfringens* ^{125}I-enterotoxin to rabbit intestinal cells. Biochem 21:4801-4807

McDonel JL (1986) Toxins of *Clostridium perfringens* types A, B, C, D and E. In: Dorner F, Drews H (eds) Pharmacology of Bacterial Toxins. Oxford, Pergamon Press, pp 477-517

McDonel JL, Duncan CL (1975) Histopathological effects of *Clostridium perfringens* enterotoxin in the rabbit ileum. Infect Immun 12:1214-1218

McDonel JL, McClane BA (1979) Binding vs. biological activity of *Clostridium perfringens* enterotoxin in Vero cells. Biochem Biophys Res Commun 87:497-504

MacLennan JD (1962) The histotoxic clostridial infections of man. Bact Rev 26(2):177-276

Melville SB, Labbe RG, Sonenshein AL (1994) Expression from the *Clostridium perfringens cpe* promoter in *C. perfringens* and *Bacillus subtilis*. Infect Immun 62:5550-5558

Mietzner RA, Kokai-Kun JF, Hanna PC, McClane BA (1992) A conjugated synthetic peptide corresponding to the C-terminal region of *Clostridium perfringens* type A enterotoxin elicits an enterotoxin-neutralizing antibody response in mice. Infect Immun 60:3947-3951

Morris JA (1987) Hypothesis: common bacterial toxins are a possible cause of the sudden infant death syndrome. Med Hypoth 22:211-222

Murrell TGC, Ingham B, Moss J, Taylor W (1987) A hypothesis concerning *Clostridium perfringens* type A enterotoxin and SIDS. Med Hypoth 22:401-413

Murrell TGC, Murrell WG, Lindsay JA (1994) Sudden infant death syndrome (SIDS): are common bacterial toxins responsible, and do they have a vaccine potential. Vaccine 12:365-368

Murrell TGC, Walker PD (1991) The pigbel story Papua New Guinea. Trends R Soc Trop Med 85:119-122

Murrell WG (1989) *Clostridium perfringens*. In: Buckle K (ed) Foodborne Microorganisms of Public Health Significance. Sydney, Australia, AIFST, pp 209-232

Murrell WG, Stewart B, O'Neill C, Siarakas S, Kariks S (1992) The role of enterotoxigenic bacteria in SIDS. Abstract of the 2nd International SIDS Conference, Sydney, Australia 1992:52

Murrell WG, Stewart B, O'Neill C, Siarakas S, Kariks S (1993) Enterotoxigenic bacteria in SIDS. J Med Micro 39:114-127

Niilo L (1975) Measurement of biological activities of purified and crude enterotoxin of *Clostridium perfringens*. Infec Immun 12:440-442

Niilo L (1980) *Clostridium perfringens* in animal disease: a review of current knowledge. Can Vet J 21:141-148

Pastan IH, Pai LH, Brinkman U, Fitzgerald DJ (1995) Recombinant toxins: new therapeutic agents for cancer. Ann NY Acad Sci 745:345-354

Pestka S, Langer J, Zoon K, Samuel C (1987) Interferons and their actions. Ann Rev Biochem 56:27-72

Ray CG, Hebestreit NM (1971) Studies of the sudden infant death syndrome in King county, Washington, II. Attempts to demonstrate evidence of viremia. Pediatrics 49:79-82

Rood JI, Cole ST (1991) Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol Rev 55:621-648

Saito M (1990) Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, foods and the natural environment in Japan. J Food Protection 53:115-118

Seto DS, Carver DH (1978) Circulating interferon in sudden infant death syndrome. Proc Soc Exp Biol Med 157:378-380

Sherman S, Klein E, McClane BA (1994) *Clostridium perfringens* type A enterotoxin induces tissue damage and fluid accumulation in rabbit ileum. J Diarrheal Dis Res 12:200-207

Skjelkvale R, Tolleshaug H, Jarmund T (1980) Binding of enterotoxin from *Clostridium pefringens* type A to liver cells *in vivo* and *in vitro*. Acta Path Microbiol Scan 88:95-102

Skjelkvale R, Uemura T (1977) Experimental diarrhea in human volunteers following oral administration of *Clostridium perfringens* enterotoxin. J Appl Bacteriol 46:281-286

Smith LDS (1979) Virulence factors of *Clostridium perfringens*. Rev Infect Dis 1:254-260

Smith WP, McDonel JL (1980) *Clostridium perfringens* type A: *in vitro* systems for sporulation and enterotoxin synthesis. J Bacteriol 144:306-311

Songer JG (1996) Clostridial enteric diseases of domestic animals. Clin Microbiol Rev 9:216-234

Spika JS, Shaffer N, Hargrett-Bean N, Collin S, MacDonald KL, Blake PA (1989) Risk factors for infant botulism in the United States. Am J Dis Child 143:828-832

Staton AN (1980) Is overheating a factor in some unexpected infant deaths? Lancet 3:1054-1057

Stephens T (1990) Animal models give clues to SIDS. J NIH Res 1:73-80

Sterne M (1981) Clostridial infections. Br Vet J 137:443-454

Stringer MF (1985) *Clostridium perfringens* type A food poisoning. In: Poriello SP (ed) Clostridia in Gastrointestinal Disease. Boca Raton, FL, CRC Press, pp 117-144

Sugii S, Horiguchi Y (1988) Identification and isolation of the binding substance for *Clostridium perfringens* enterotoxin on Vero cells. FEMS Microbiol Let 52:85-90

Sugimoto W, Takagi M, Ozutsumi K, Harada S, Matsuda M (1988) Enterotoxin of *Clostridium perfringens* type A forms ion-permeable channels in a lipid bilayer membrane. Biochem Biophys Res Commun 156:551-556

Sugiyama H (1986) Mouse models for infant botulism. In: Zak O, Sande MA (eds) Experimental Models in Antimicrobial Chemotherapy. New York, NY, Academic Press, pp 73-91

Thach BT, Davies AM, Koenig JS (1988) Pathophysiology of sudden upper airway obstruction in sleeping infants and its relevance for SIDS. Ann N Y Acad Sci 533:314-328

Todd ECD (1989) Cost of acute bacterial foodborne disease in Canada and the United States. Int J Food Microbiol 9:313-326

Tracey KJ, Cerami A (1993) Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol 9:317-343

Tsai CC, Riemann HP (1975a) Food poisoning signs in mice induced orally by *Clostridium perfringens* type A enterotoxin. J Formosan Med Assoc 74:310-315

Tsai CC, Riemann HP (1975b) Oral infection and food poisoning in mice by enterotoxigenic *Clostridium perfringens* type A. J Formosan Med Assoc 74:361-371

Uemura T, Sakaguchi G, Itoh T, Okazawa K, Sakai S (1975) Experimental diarrhea in cynomolgus monkeys by oral administration with *Clostridium perfringens* type A viable cells or enterotoxin. Japan J Med Sci Biol 28:165-177

Valdes-Dapena M (1980) Sudden infant death syndrome: a review of the medical literature 1974-79. Pediatrics 66:597-614

Valdes-Dapena M (1983) The morphology of the sudden infant death syndrome: an overview. In: Tildon JT, Roeder LM, Steinschneider A (eds) Sudden Infant Death Syndrome. New York, Academic Press, pp 169-182

Van Damme-Jongsten M, Weners K, Notermans S (1989) Cloning and sequencing of the Clostridium perfringens enterotoxin gene. Antonie von Leeuwnhoek J Microbiol 56:181-190

Verrier RL, Kirby DA (1988) Sleep and cardiac arrhythmias. Ann N Y Acad Sci 533:238-251

Wallace FM, Keller AM, Lindsay JA (1997) Cytokine response after administration of *Clostridium perfringens* enterotoxin in the murine model. 97th ASM General Meeting, Miami Beach, Florida, Abstract P-028, pp 441

Weiss KF, Strong DH, Groom RA (1965) Mice and monkeys as assays for *Clostridium perfringens* food poisoning. Appl Microbiol 14:479-485

Whitaker JR, Granum PE (1980) The role of amino groups in the biological and antigenic activities of *Clostridium perfringens* type A enterotoxin. J Food Biochem 4:201-217

Wieckowski EU, Wnek AP, McClane BA (1994) Evidence that an ~50-kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound *Clostridium perfringens* enterotoxin. J Biol Chem 269:10838-10848

Wilkinson MA (1992) The sudden infant death syndrome in florida: an epidemiological, pathological and microbiological study. M.S. Thesis, University of Florida

Willinger M (1989) SIDS: a challenge. J NIH Res 1:73-80

Wnek AP, McClane BA (1983) Identification of a 50,000 Mr protein from rabbit brush border membranes that bind *Clostridium perfringens* enterotoxin. Biochem Biophys Res Com 112:1099-1105

Wnek AP, McClane BA (1986) Comparison of receptors for *Clostridium perfringens* type A and cholera enterotoxins in isolated rabbit intestinal brush border membranes. Microb Pathog 1:89-100

Wnek AP, McClane BA (1989) Preliminary evidence that *Clostridium perfringens* type A enterotoxin is present in a 160,000-Mr complex in mammalian membranes. Infect Immun 57:574-581

Wnek AP, Stouse RJ, McClane BA (1985) Production and characterization of monoclonal antibodies against *Clostridium perfringens* type A enterotoxin. Infect Immun 50:442-448

Wojciechowski LM (1995) The cloning and characterization of the *Clostridium perfringens* type A 8-6 enterotoxin gene. Ph.D. Dissertation, University of Florida

Zylke J (1989) Sudden infant death syndrome: resurgent research offers hope. JAMA 262:1565-1566

BIOGRAPHICAL SKETCH

Andreas M. Keller was born in Nürnberg, Germany. In 1967 his parents immigrated to Brazil, and he was raised in São Paulo and Rio de Janeiro. He graduated with a B.S. degree in agronomy in 1988 from the Federal Rural University of Rio de Janeiro, majoring in food technology. He received his M.S. degree in foods and human nutrition from Eastern Michigan University in 1990. In fall of 1990 he enrolled in the Food Science and Human Nutrition Department's doctoral program at the University of Florida. During his tenure at UF he worked for the University Division of Housing as Resident Manager for Maguire Village, University Village South and Tanglewood. He was an active member of Student Government serving the UF students as a Family Housing Senator, Chairman of Mayor's Council and as Student Senate President, and was awarded two University of Florida Presidential Recognitions, and an Outstanding Student Senator award for his outstanding achievements. Upon completion of his degree he plans to pursue a career as a food microbiologist with an international food company.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James A. Lindsay, Chair
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Douglas L. Archer
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sean F. O'Keefe
Associate Professor of
Food Science and
Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Mark L. Tamplin
Associate Professor of
Family Youth and Community
Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ramon C. Littell
Professor of Statistics

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1997

Dean, College of Agriculture

Dean, Graduate School