PURIFICATION AND CHARACTERIZATION OF *CLOSTRIDIUM SORDELLII* TOXINS HT AND LT AND COMPARISON TO TOXINS A AND B OF *CLOSTRIDIUM DIFFICILE*

by

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FOREWORD

This dissertation contains five sections. Section II is a review of the literature that serves as an introduction to the research problem. Sections III and IV were written in the form of two papers for publication with the concurrence of my committee. The first paper (Section III) has been published, the other will be submitted. Section V is an overall discussion and summary.

The titles of the papers are as follows:

Martinez, R.D., and T.D. Wilkins. 1988. Purification and Characterization of Clostridium sordellii Hemorrhagic Toxin and Cross-reactivity with Clostridium difficile Toxin A (Enterotoxin). Infect. Immun. 56:1215-1221.

Martinez, R.D., and T.D. Wilkins. 1989. Comparison of Clostridium sordellii Toxins HT and LT to Toxins A and B of Clostridium difficile. To be submitted to Infection and Immunity.

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TABLE OF CONTENTS

Section I. Introduction - Statement of research problem

Treatment with antibiotics can cause a variety of untoward effects, including diarrhea and other gastrointestinal disturbances. About twenty percent of the hospitalized patients taking antibiotics are affected by diarrhea that can progress into a life-threatening disease known as pseudomembranous colitis (PMC). Clostridium sordellii was once believed to be the cause of PMC in humans. This was because the toxicity of stool filtrates of patients with PMC was neutralized by C. sordellii antitoxin; however, this bacterium was never isolated from the stool samples of these patients. Clostridium difficile, not C. sordellii, was found to be the causative agent of PMC in humans, and it was shown that C. difficile produced a toxin that was neutralized by C. sordellii antitoxin. Thus, the fortuitus cross-reaction between C. difficile toxin and C. sordellii antitoxin contributed to the discovery of the cause of PMC.

Two toxins, A and B, have been purified from culture filtrates of toxigenic strains of C. difficile. Fundamental information has been obtained about the biochemical and immunological properties of these toxins. In addition, it has been proven that C. difficile and its toxins are implicated in the pathogenesis of the disease. Although cross-neutralization experiments demonstrated that

the toxins produced by C. sordellii are similar to the toxins of C. difficile, the former were not studied to the same extent. The purpose of my research was to characterize the toxins of C. sordellii and compare their properties with the toxins of C. difficile.

Section II. Literature Review

Role of Clostridium sordellii in disease.

A. Source of the organism. Clostridium sordellii was first described by Sordelli who isolated it from a case of human gas gangrene (164) and observed that the cultural characteristics of this organism differed from those of others commonly involved in this disease. Because of the similarities to Bacillus oedematiens (Clostridium novyi) and to Bacillus sporogenes (Clostridium sporogenes), he named the previously undescribed organism as "Bacillus oedematis sporogenes". In 1927 Meleney et al., described an anaerobic bacillus isolated from a case of post-operative gas gangrene (114). Unaware of the findings by Sordelli, the authors believed that they had described a new species and named it Clostridium oedematoides. Later that year Hall and Scott published a study comparing the strains isolated by Sordelli with those of Meleney and collaborators. Cultural and biochemical comparison of the different isolates revealed no fundamental differences; this led to the conclusion that both organisms were identical and they suggested the use of the binomial Bacillus sordellii (77). Because of the anaerobic nature of the organism this name was changed to Clostridium sordellii.

Little is known about the distribution of C. sordellii in the environment, although several reports indicate that soil

is it principal habitat (162, 192). It can be isolated from the intestinal content of animals (28, 192), and it is usually either absent from the natural flora of humans or is present in very low numbers (61, 70, 162). Nevertheless, C. sordellii can be isolated from a variety of clinical specimens, especially those exposed to soil or fecal contamination. (70, 122, 162, 192).

B. Gas gangrene. Clostridium sordellii is one of several species of the genus Clostridium commonly identified as a cause of gas gangrene (clostridial myonecrosis) in humans and other animals (75, 76, 77, 84, 110, 111, 114, 115, 161, 162, 163, 164, 165, 186, 187).

Gas gangrene is an acute invasion of healthy living tissue. The organisms capable of causing this disease are so widely distributed in nature that their presence in any wound is the rule rather than the exception (111, 187, 192). An essential factor in the genesis of gas gangrene is trauma, particularly trauma producing deep and lacerated wounds of muscle. Another important prerequisite for this type of clostridial infection is that there must exist a reduced oxygen/reduction potential in the area of the wound (111, 187, 192). In such deep wounds there are always areas of ischemia, shut off from all contact with oxygen, and this provides ideal conditions for the initiation of an

infection.

In humans, the lesions of the areas infected by C. sordellii are characterized by an extensive edema with patchy hemorrhage (111, 114, 162, 192). The clinical picture is accompanied by severe pain in the area of the lesion, hypotension, elevation of temperature and leukocytosis. At the final stages of the infection the patients go into a profound prostration with circulatory failure (76, 80, 111, 114, 162, 163).

Several reports have incriminated C. sordellii as a causative agent in cases of postpartum vulvar edema and toxic shock (80, 110, 163). Most of the infections were characterized by massive edema and exhibit the typical symptoms associated with gas gangrene infection by this organism. The majority of these patients had midline episiotomies with extensions into the rectum, which greatly increase the risk of contamination with fecal material. Presumably, the presence of traumatized tissue with a compromised blood supply could have created appropriate conditions for growth of potentially pathogenic anaerobic organisms, in these instances C. sordellii.

C. Enterotoxemia. Clostridium sordellii also has been implicated as a cause of gastrointestinal disease in a variety of domestic animals (4, 5, 28, 32, 79, 136, 147).

Brooks et al., reported on the isolation of C. sordellii from the intestinal contents of cows that apparently died as a result of enterotoxemia (32). Filtrates from the intestinal contents were found to be toxic, and the toxicity was neutralized by C. sordellii antiserum, suggesting that this organism and its toxins were the cause of death. Hibb et al., reported a case of enterotoxemia in foals due to C. sordellii (79). The organism was isolated from the intestinal content of diarrheic animals, but neither the feces nor the intestinal content were evaluated for the presence of C. sordellii toxins. Pathological observations revealed extensive hemorrhage of the small intestine, which is consistent with observations by other investigators. In other studies C. sordellii has been listed as a causative agent of inflammatory lesions of the gastrointestinal tract of cattle (4, 28). Pure cultures of a toxigenic strain of C. sordellii were used in experiments designed to confirm the role of this organism in gastrointestinal disease of cattle (6). Oral challenge of calves with suspensions of C. sordellii cells resulted in passage of soft, mucous feces streaked with blood. Animals that were challenged with the culture filtrate of the toxigenic C. sordellii passed pasty feces that were streaked with blood. Pathological observations of the animals challenged with the culture filtrate included inflammation, edema and erosion of the

mucosal surface of the jejunum, ileum and cecum. Basically, the damage induced by the cell-free preparation was similar to that induced by challenge with live organisms (6).

Previous studies of experimental strangulation obstruction have implicated C. sordellii toxins as responsible for the lethal effect of the fluid in strangulated intestines of dogs. Bornside et al. observed that toxic substances contained in the fluid of strangulated dog intestines could be neutralized by C.sordellii antitoxin, and that mice passively immunized with the antitoxin were protected from the lethal effects of the toxic substance (26). Although speciation of the clostridia isolated from the strangulated loops was not performed these investigators concluded from the neutralization experiments, that the toxic substance was a C. sordellii toxin. Subsequent experiments by Allo, demonstrated that C. sordellii can be isolated from strangulated dog intestinal loops (3). Culture filtrates of these C. sordellii isolates caused diarrhea and colitis when injected intracecally to hamsters, as did loop fluid-filtrates.

Clostridium difficile and pseudomembranous colitis.

Pseudomembranous colitis (PMC) is a severe gastrointestinal disease characterized by exudative plaques that often form a pseudomembrane on the intestinal mucosa

(69, 113, 142). When examined microscopically, this pseudomembrane consisted of detached epithelial cells, fibrin, mucus, cellular debris, and polymorphonuclear cells (31, 69, 113, 142). The lesions may occur in any part of the gastrointestinal tract, however, the most commonly involved site is the colon. This condition was initially described in the preantibiotic era when it was frequently found in association with intestinal surgery (15, 69). With the advent of antibiotics, the disease has been recognized as a complication of antimicrobial use. In the 1950's and 1960's the drugs most frequently implicated were chloramphenicol, tetracycline, and neomycin (12, 30, 31, 145, 166, 179). During this time most cases were attributed to Staphylococcus aureus (7, 43, 87, 90, 166), although its role in the disease was never confirmed. In the 1970's cases of PMC became much more common as a direct result of treatment with broad-range antibiotics, although it was noticed that clindamycin causes this complication much more often per time used (13, 18, 30, 31, 41, 42, 44, 45, 55, 64, 65, 73, 103, 142, 179). Like virtually any orally administered antibiotic, clindamycin can cause a variety of untoward gastrointestinal effects, including a profuse diarrhea that can develop into PMC. Symptoms may be prolonged and debilitating, with diarrhea persisting for several weeks (30, 31, 44, 55, 64, 73, 102, 179) and high

mortality rates have been observed in seriously ill patients (41, 42, 44, 55, 64, 65, 69, 93, 113, 135, 142). Filtrates of stools from patients with PMC contained a cytophatic and lethal substance, that was suspected to be involved in the disease (93). It was also known at that time, that treatment of hamsters with some antibiotics induced a disease that had some similarity to PMC in humans. When hamsters were treated with clindamycin they developed a fatal enterocolitis (1, 15, 36, 81, 104, 143, 148, 150, 160). Content of the cecum of these animals were cytophatic for tissue cultured cells, further implicating a common mechanism between human PMC and antibiotic-induced cecitis in hamsters. Logically, the hamster disease was chosen as a model system for the study of PMC.

First attempts to define the pathological mechanism in this disease utilized a series of experiments designed to detect a transferable agent (17, 148). When cecal content from hamsters with antibiotic-induced cecitis was injected intracecally into healthy animals, they developed the disease, thus demonstrating that a transferable agent was involved. Since the cytophatic effect on tissue-cultured cells was similar to the effect produced by viruses, viral etiology was suspected. However, the substance responsible for the toxic effects could not be propagated or filtered through a PM-10 ultrafilter. Therefore, this toxic substance

was larger than 10,000 daltons but smaller than a virus, which led many investigators to suspect that it was a bacterial toxin. Subsequent experiments showed that the cytophatic and lethal activities found in stool samples of patients with PMC were neutralized by gas gangrene polyvalent antitoxin (14, 17, 94). This gas gangrene antitoxin was a mixture of antitoxins made to the culture filtrates of each of the clostridia that are commonly associated with gas gangrene: C. histolyticum, C.novyi, C. perfringens, C. septicum, and C. sordellii. The C. sordellii antitoxin was found to have all of the neutralizing activity (2, 37, 94, 148, 149, 191), which led to the premature conclusion that PMC was a C. sordellii-colitis. Nevertheless, it was confusing that this organism was not isolated from the stools of patients with PMC, despite the fact that C. sordellii is relatively easy to cultivate. Also disturbing was the observation that very few strains of C. sordellii were able to produce a toxin in vitro (15, 150).

Information implicating C. difficile as the causative agent of PMC came from stool cultures of both hamsters and humans with PMC. Selective media incorporating either antibiotics or alcohol inhibited most of the normal flora, and C. difficile was easily isolated from the fecal specimens (18, 29, 63, 65, 66, 95, 150). Intracecal injections of C. difficile or the culture filtrates these

isolates were shown to produce the typical pathological damage observed in hamsters with the clindamycin-induced disease. Further evidence came from the observation that culture filtrates of C. difficile contained a toxin which produced cytopathic changes in tissue cultured cells, and that this toxic activity was neutralized by C. sordellii antitoxin (9, 16, 17, 37, 38, 50, 95, 144, 149). Therefore, the discovery of C. difficile as a causative agent of PMC resulted from the fortuitus cross-reaction between C. difficile toxin and C. sordellii antitoxin.

The antigenic cross-reactivity between the toxins produced by C. difficile and by C. sordellii has been confirmed in many subsequent studies. Several investigators have shown that antisera against the toxic cultures filtrates of either species neutralizes the action of toxins present in the culture filtrate of the other organism, although usually in a less potent manner (9, 37, 50, 189). Heterologous toxin-antibody reactions could be readily dissociated by simple dilution or by the addition of ammonium sulfate (37).

The information obtained from cross-reactivity experiments were interpreted to signify a partial antigenic relationship between the toxins of C. difficile and those of C. sordellii, and supported the idea that these toxins were similar in many respects. However, these studies also revealed that differences exist between these toxins, and

that further studies were required to evaluate in detail the nature and extent of their similarities.

Clostridium difficile toxins.

The discovery of C. difficile as the cause of antibiotic-associated pseudomembranous colitis prompted many investigators to study the toxins produced by this organism. At that time, only a single toxin was believed to be produced by the organism. The production of a second toxin was reported in 1981 by Taylor et al., and by Banno et al. (11, 178). It was shown by these investigators that the two toxins could be separated by anion exchange chromatography. Taylor et al. designated first toxin to elute from the anion exchange column as toxin A and they referred to the second toxin as toxin B (178). Banno et al. used the designation D-1 and D-2 for toxins A and B, respectively (11). Both toxins were shown to be cytotoxic, but unlike toxin B, toxin A elicited a positive response in the rabbit ileal loop assay (11, 176, 178). This indicated that toxin A was an enterotoxin.

Compelling evidence implicates the two toxins in the development of the disease. Both toxins are consistently detected in the stools of patients with PMC (31, 96) and are highly lethal when injected into animals (11, 105, 108, 176). In addition, it has been shown that protection of

experimental animals against the disease requires vaccination against both toxins (56, 88, 100), and that the intestinal pathology caused by the purified toxins in hamsters resembles the pathology observed in the disease (100, 105, 106, 109).

Consequently, much of the work on C. difficile disease has centered on the purification and characterization of toxins A and B.

A. Toxin A. Several methods have been reported for the purification of toxin A. Most of the procedures included a concentration step, gel filtration and ion-exchange chromatography (11, 105, 108, 176, 178). The ion-exchange step has been used extensively because it separates toxins A and B effectively. Other methods for the purification of toxin A included preparative electrophoresis, chromatofocusing using FPLC, immunoaffinity chromatography, and thermal-affinity receptor chromatography (92, 105, 107, 150,). The method described by Sullivan et al., consisted of ultrafiltration, DEAE anion exchange chromatography, and precipitation in acetate buffer at pH 5.5 (176). Toxin A purified by this method has been shown to be homogeneous by several criteria.

Toxin A is a large, heat labile, hydrophobic protein with an estimated native molecular weight of 440,000-600,000 and

an isoelectric point of 5.2-5.7 (10, 11, 92, 105, 108, 176, 178, 189). The toxin is stable from pH 4 to 10, and is inactivated by proteolytic enzymes (11, 105, 176). Under denaturing conditions the toxin has a molecular weight of about 300,000 and does not dissociate to subunits (10, 107, 176, 178). The gene for toxin A has been sequenced, and encodes for a mature polypeptide with a calculated molecular weight of 308,000 daltons, which supports the observations from SDS-PAGE. Rihn et al. reported that toxin A has a molecular weight of 52,000 (151), however, this observation has not been confirmed by others, and this work disagrees with the size estimated from the nucleotide sequence.

Toxin A contains high amounts of glutamic acid, aspartic acid, and glycine, and low amounts of sulfur-containing amino acids. Toxin A also contains high amount of the hydrophobic amino acids leucine, isoleucine, and valine (10, 105, 107, 108). The toxin does not appear to contain hexose, pentose, nor any detectable amino-sugar or phosphorous (11, 176).

Toxin A possesses three biological activities: cytotoxicity, lethality, and potent enterotoxicity. Toxin A is cytotoxic to all mammalian cell lines which have been tested (31, 38, 46, 47, 105), and a rounding effect is also caused in all cell lines. Nanogram (2ug/Kg) amounts of toxin A are lethal to mice by intraperitoneal or intravenous

injection (10, 11, 105, 108, 176). Injection of toxin A into the skin of rabbits produces erythematous and hemorrhagic lesions and increases vascular permeability (10, 106, 178).

The enterotoxic activity of toxin A was initially detected using ligated intestinal loops in rabbits (11, 177). The fluid which accumulates in the ileal loops treated with toxin A is hemorrhagic, and an intense inflammatory response occurs with extensive damage to the gut mucosa (100, 102, 105, 106, 109, 121). This enterotoxic response is different from the one observed with the enterotoxins of Vibrio cholerae and Escherichia coli, in which a "ricewater" fluid is produced, and little or no tissue damage is observed. Toxin A also causes a positive fluid response in rabbit colonic loops, where the tissue damage is not as extensive as in the ileal loops (102, 121). Toxin A also causes fluid to accumulate in the intestine of infant mice after intragastric challenge (106). Intracecal injection of toxin A in hamsters causes moderate hemorrhage, acute inflammation, necrosis, fluid accumulation and death (100). Toxin A given intragastrically to hamsters also causes the same symptoms (109).

Hamsters are at least ten-fold more sensitive to toxin A given intragastrically than mice or rats, which may be indicative of differences in the nature and/or number of receptors in the intestines. Krivan et al. have shown that

toxin A binds to the trisaccharide Galα1-3Galβ1-4GlcNAc (91), which is also found on rabbit erythrocytes and bovine thyroglobulin. Binding of toxin A to this trisaccharide is enhanced at 4°C, and this temperature effect has been used to purify the toxin by thermal-affinity chromatography with bovine thyroglobulin as the ligand (92). Toxin purified by this method has properties identical to those of toxin A purified by conventional methods. Whether or not this trisaccharide is a physiological receptor for toxin A remains to be determined.

B. Toxin B. Sullivan et al. also developed a method for the purification of toxin B. The method consisted of ultrafiltration, hydrophobic interaction chromatography, anion-exchange chromatography, and immunoaffinity chromatography (176). Like toxin A, toxin B is extremely large and has a native molecular weight of 360,000-470,000 with an isoelectric point of 4.1-4.5 (10, 11, 105, 108, 176, 178). Under denaturing conditions the toxin has a molecular weight in excess of 250,000 daltons (10, 105, 107, 176). Toxin B is more unstable than toxin A. Homogeneous preparations of toxin B have been shown to contain components of varying molecular weight which are immunologically related to the large form of the toxin (175, 183). It has been reported that toxin B is composed of

50,000 molecular weight subunits (140). However, this polypeptide seems to be a contaminant since it cannot be derived from the 250,000 dalton protein, it is not necessary for toxic activity (112), and in addition it is produced by nontoxigenic strains of C. difficile.

Toxin B is readily inactivated by pH extremes, oxidation, low or high temperature, and is susceptible to proteases (10, 105, 176, 178). Like toxin A, toxin B contains high amounts of glutamic acid, aspartic acid, glycine, leucine, isoleucine and valine; and low amounts of sulfur-containing amino acids (10, 105, 108), and does not contain phosphorous, hexose or pentose. Toxin B is about a 1000-fold more active than toxin A against monolayer cultures of Chinese Hamster Ovary cells, with a tissue culture death (TCD_{100}) dose of about 1 pg (10, 105, 108, 176). Like toxin A, toxin B is active against all mammalian cells tested and induce an identical rounding effect (31, 38, 46, 105, 180, 181). When injected intraperitoneally into mice is lethal in nanograms amounts (10, 11, 105, 108, 176) however, toxin B is devoid of enterotoxic activity (10, 11, 109, 176, 178). Toxin B given intragastrically to hamsters does not cause a response, but when given with low amounts of toxin A the animals die (109). Also, intracecal injection of toxin B in hamsters causes hemorrhage and inflammation in the mucosal wall (100). Thus seems that once the tissue is disrupted by

either toxin A or mechanical trauma, toxin B can cause tissue damage and even death. In addition, toxin B produce erythematous, necrotic lesions, and increased vascular permeability when injected intradermally into rabbit skin (10, 105, 106, 178).

Early studies on the Clostridium sordellii toxins.

The toxigenic nature of Clostridium sordellii was recognized by Sordelli (164, 165). He developed an antitoxin to a strain isolated from a case of human gas gangrene, and recommended that C. sordellii antitoxin should be included as part of the therapy for gas gangrene (165).

Several investigators observed that only strains of C. sordellii producing an edema factor were pathogenic to laboratory animals (77, 101, 114, 115). Culture filtrates containing this edema factor were dermonecrotic and lethal to mice by intravenous injection (8, 101, 114, 115). The lethal toxin was clearly distinct from enzymes that were produced in large amounts by non-pathogenic strains. In addition, the toxin was shown to be thermolabile, and could not be neutralized by antitoxins against other pathogenic Clostridium (8, 114, 164, 165). Intramuscular injections of crude culture filtrate from toxigenic strains of C. sordellii induced tissue damage similar to the changes occurring in animals infected with the live organism.

Intravenous injection of crude culture filtrate from a toxigenic strain into mice, caused congestion, hemorrhage and necrosis of the major organs (101). Therefore, the systemic and local manifestations of infection by C. sordellii were attributed to the lethal toxin. Saissac and Raynaud were able to extract a toxin from vegetative cells after consecutive washings with distilled water and suspension of the washed cells in saline solution at 4°C (155). This toxin was lethal to mice by intraperitoneal injection.

The production of two distinct toxins by C. sordellii was first described in 1969 by Arseculeratne et al. Following a procedure similar to the one described by Saissac and Raynaud, these investigators were able to extract a lethal, edema-producing toxin from vegetative cells, while a hemorrhagic toxin was extracted from sporulating cells (8). The edema-producing toxin was shown to be more lethal than the hemorrhagic toxin, therefore the toxins were designated LT (Lethal Toxin) and HT (Hemorrhagic Toxin) respectively. The toxins were shown to be independent since the lesions induced by each one when injected intradermally to guinea-pigs were clearly distinguishable. In addition, heat stability studies showed a difference in heat resistance between HT and LT, further suggesting that two independent toxins were produced by the organism (8).

During the 1970's there were no further reports on the toxins of C. sordellii. However, as discussed earlier, it was discovered during this period that C. sordellii antitoxin neutralizes the toxins of C. difficile (2, 37, 94, 148, 149, 191), indicating that the toxins produced by these organisms are antigenically related.

In the early 1980's several reports indicated that C. sordellii and it toxins were the cause of some cases of gastrointestinal disease in domestic animals (see section on enterotoxemia). C. sordellii was isolated in high numbers from animals with hemorrhagic diarrhea as well as from inflammatory lesions of the gastrointestinal tract (4, 5, 136, 147). These findings provided the impetus for new investigations on the toxins of C. sordellii. Al Mashat and Taylor simulated the natural disease by challenging animals with either a pure culture of a toxigenic C. sordellii isolated from a case of hemorrhagic enteritis or with the culture filtrate of this strain (6). Nakamura et al., observed that culture filtrates from toxigenic strains of C. sordellii were cytotoxic to tissue-cultured mammalian cells (123, 124), and that the toxins induced the tissue-cultured cells to round-up. This effect on tissue cultured cells is similar to the effect observed with the toxins of C. difficile. In addition, crude culture filtrates were shown to elicit accumulation of hemorrhagic fluid in the rabbit

ileal loop (194). In 1985 Yamakawa et al. reported on the separation, by gel filtration and anion exchange chromatography, of two antigenically distinct toxins from the culture filtrate of C. sordellii (193). Like toxin A of C. difficile, the toxin to elute first from the anion exchange column possessed both, cytotoxic and enterotoxic activity, while the second toxin, like toxin B of C. difficile, was shown to be cytotoxic but not enterotoxic. Although the results clearly indicated similarities between the partially purified toxins and the toxins of C. difficile, their antigenic relationship was not studied, and in addition, these authors did not mention previous studies that documented the cross-reactivity between these toxins.

During the course of the experimental work discussed in this dissertation, Popoff reported the purification of the lethal toxin (LT) of C. sordellii, and demonstrated that this toxin is similar to toxin B of C. difficile. His purification scheme for toxin LT involved ion-exchange chromatography, gel filtration, and hydroxyapatite adsorption chromatography (137). The toxin has a molecular weight of about 250,000 and a pI of 4.5. Like toxin B of C. difficile, toxin LT is cytotoxic and lethal to mice by intraperitoneal injection. Like with toxin B, a 50,000 dalton protein was observed with the major 250,000 protein band. Popoff assumed that this was a subunit toxin LT,

however, antiserum against this 50,000 daltons protein did not neutralize the toxic activity of the native LT. In addition, a similar protein is produced by nontoxigenic strains which suggests that this protein is a contaminant. It is interesting that a similar molecular weight "contaminant" protein has been associated with both, toxins B and LT, and therefore a role for these proteins in toxicity cannot be completely ruled out at this moment.

Further comparison of these toxins are presented in the next two sections.

Other biologically active factors produced by *Clostridium sordellii*.

In addition to the hemorrhagic and lethal toxins, *Clostridium sordellii* produces a variety of biologically active substances that do not appear to play an important role in infections caused by this organism and all of these substances are also produced by non-toxigenic strains.

C. sordellii produce a lecithinase that is antigenically and pharmacologically identical to the lecithinase produced by *C. bifermentans* (99). The lecithinases of these two clostridia are also serologically and biochemically related to the lecithinase C (alpha toxin) of *C. perfringens* (119, 120), although they are considerably less toxic than the alpha toxin. The relationship seems to be remote. A

lysolecithinase is also produced (116).

C. sordellii also produces a hemolysin which is distinct from the lecithinase. This is one of a family of oxygen-labile hemolysins which have similar properties and are serologically related. These include the theta toxin of C. perfringens, streptolysin O, tetanolysin, the hemolysin of C. histolyticum, the hemolysin of the non-pathogenic C. bifermentans, and lytic toxins produced by organisms of the genera Bacillus and Listeria (20). Like the other oxygen-labile hemolysins, it is inactivated by simple exposure to air, and reactivation may be obtained by treatment with reducing agents (72, 141). However, too long an exposure to air will inactivate it permanently.

The neuraminidase produced by C. sordellii has no pathological activity, and is produced in large amounts by some nonpathogenic strains (153, 188). A deoxyribonuclease, hyaluronidase, and proteases are also produced by C. sordellii (126), however, there is no evidence indicating that these enzymes play a role in the pathogenicity of the organism.

Cross-reactivity between other bacterial toxins.

Sharing of similar toxins (and therefore of toxin genes) between different species, and even different genera of bacteria is common. For instance, several other bacterial

species besides Escherichia coli produce a heat-stable (ST) enterotoxin (71). These include functionally and structurally related enterotoxins produced by Yersinia enterocolitica, Klebsiella pneumoniae, Enterobacter cloacae, Aeromonas hydrophila and some species of Salmonella. A more widespread distribution of closely related toxins is observed with the oxygen-labile hemolysins, which includes lytic toxins produced by members of the Bacillus, Clostridium, Listeria, and Streptococcus genera (20).

The sharing of antigenic, as well as functional properties between bacterial toxins has been very useful in the identification and characterization of suspected or newly identified toxins. For example, the characterization of cholera toxin has provided a model for the discovery and characterization of other cholera-like toxins (57, 58).

Detailed comparison of similar toxins can also provide important information because the portions of the molecules which are conserved are usually the active and binding sites. Diphtheria toxin and Pseudomonas aeruginosa exotoxin A have been shown to possess identical enzymatic properties (118). Both toxins catalyze the NAD-dependent ADP-ribosylation of elongation factor 2 (EF-2) within mammalian cells, which results in inhibition of protein synthesis and ultimately of cell death. Despite this observation, the toxins were considered immunologically distinct because

cross-reactivity could not be readily demonstrated. However, Sadoff et al. showed that diphtheria and exotoxin A share an antigenic determinant which is readily accessible in exotoxin A but is exposed in diphtheria toxin only after treatment with trypsin and a reducing agent (154). This finding was recently confirmed. Comparison of the amino acid sequences of diphtheria toxin and exotoxin A revealed the existence of significant identity between the active sites of both toxins (35).

Cross-reactive toxins can differ also to some extent in their physicochemical and biological properties, as well as in their susceptible hosts. These differences may result from minor structural changes due to genetic drift. As with the conserved portions, structural variations also can be helpful in mapping at the molecular level the regions involved in toxic activity.

The following is a brief overview of some of the best characterized cross-reactive toxins.

A. Cholera toxin and Escherichia coli LT toxin. Vibrio cholerae causes diarrhea in man, and enterotoxigenic strains of Escherichia coli cause a somewhat similar diarrheal disease in man and animals (84, 146, 152).

Cholera is characterized by an acute enteritis following bacterial colonization of the small intestine. Massive fluid

loss frequently leads to severe dehydration and shock. The clinical manifestations of cholera are the result of an extracellular enterotoxin designated cholera toxin (146). This is one of the best studied proteins with respect to structure, function, and biological activity. Cholera toxin (MW 84,000), is composed of five identical, non-covalently linked B subunits (MW 11,600 each), and one A subunit. The A subunit consists of an A_1 peptide (MW 22,000) and an A_2 peptide (MW 6,000) joined by a disulfide bond (59). The five B subunits are responsible for specific binding of the toxin to its receptor GM1 ganglioside on target cell membranes (59, 146). The A_1 peptide is responsible for the biological activity of the molecule, and has been shown to ADP-ribosylate the GTP-regulatory protein of the adenylate cyclase system, thus increasing the intracellular levels of cAMP (67). The increase in cAMP leads to the rapid secretion of electrolytes into the lumen of the human small bowel, resulting in fluid losses as high as 1 liter per hour.

Certain strains of Escherichia coli have been identified as causative agents of a severe cholera-like disease in humans and domestic animals (146, 152). The heat-labile enterotoxin (LT) produced by enterotoxigenic strains of E. coli is one of the toxins that has been associated with the disease, and it is structurally and functionally similar to cholera toxin (48, 152). E. coli LT has a molecular weight

of approximately 91,000, and is composed of five identical B subunits (MW 11,800 each), and one A (MW 29,000). The A subunit can be converted by limited proteolysis into an enzymatically active peptide, A1, linked by a disulfide bond to an A2-like chain. LT toxin activates adenylate cyclase by the same mechanism as cholera toxin, and as with cholera toxin, exposure of cells to E. coli LT results in an increase of intracellular cAMP (68, 146, 152).

Immunological studies have shown that, in addition to the similarities in subunit structure and in their mechanism of action, cholera toxin and LT are antigenically related (39, 40, 48, 54, 57, 58, 74, 89, 146). Antisera produced against cholera toxin inhibits the stimulatory effect of cholera toxin and LT on intestinal secretion, and reciprocal neutralization has been demonstrated (48, 54, 89, 146). The nucleotide sequences of the genes of subunits A and B of cholera toxin and LT have been determined and the amino acid sequences have been deduced. In the entire A and B regions, the identity between cholera toxin and LT was 78% at the nucleotide level and 79% at the amino acid level (195). This sequence comparison supports the information obtained by immunological studies, and indicates that the toxins possess considerable, but far from complete identity. The cholera/coli family of enterotoxins now includes toxins produced by Aeromonas hydrophila, Pleisomonas, Salmonella

typhimurium, Vibrio mimicus, and, Campylobacter jejuni (57, 58), which still have to be fully characterized. It will be interesting to know how these toxins relate to cholera toxin and LT with respect to antigenic divergence.

B. Shiga and shiga-like toxins. Shigellosis is an acute infectious enteritis afflicting humans and other primates (49). The organisms capable of producing disease belong to four species of the genus Shigella (S. dysenteriae, S. flexneri, S. boydii and S. sonnei). All four species appear to be capable of causing the severest form of the disease, known as bacillary dysentery (49, 86). These Shigella spp. elaborate a toxin designated as shiga toxin that has cytotoxic, lethal and enterotoxic activities, and has an important role in the development of shigellosis (86, 127). The toxin has an approximate molecular weight of 70,000 and is composed of an A subunit (MW 32,000) and five copies of a B subunit, each one with a MW of 7,700 (127, 134). Despite the structural reminiscence to cholera toxin, the toxins are not related. The A subunit must be activated by proteolysis to form an enzymatically active A1 fragment. This fragment inhibits protein synthesis by cleaving a N-glycosidic bond of an adenine from the 5'-terminus of the 28S ribosomal RNA of eukaryotic 60S ribosomal subunits (53, 156). Thus, this toxin has a completely different mode of action from cholera

toxin.

Antiserum against the toxin produced by S. dysenteriae neutralizes the biological activities of the shiga-like toxins produced by other Shigella spp. indicating that the toxins are antigenically related (86, 127). However, it has been shown that the production of shiga-like toxins is not limited to the genus Shigella. Enteropathogenic and enterohemorrhagic strains of Escherichia coli may cause intestinal disease by producing shiga-like toxins (127, 128, 129, 130). It is now known that some strains of this organism produce two kinds of toxins designated, shiga-like toxin I (SLT-I) and shiga-like toxin II (SLT-II) (127). Shiga toxin and shiga-like toxins possess similar physicochemical properties, and their enzymatic modes of action have been shown to be identical (53, 127, 156).

The properties of SLT-I are indistinguishable from those of shiga toxin, and in addition, its biological activities are neutralized by a reference shiga antitoxin (127, 128, 129, 130). Although SLT-II has physicochemical and biological properties similar to those of shiga toxin and SLT-I, its biological activities are not neutralized by antiserum against these toxins (127, 131, 173). The genes for the shiga-like toxins were cloned recently (125, 172). Comparison of the nucleotide sequences for shiga toxin and SLT-I toxin revealed that the genes have an identity of

greater than 99%, and amino acid sequence analyses showed that shiga toxin and SLT-I are basically identical toxins (83). Comparison of the sequences for SLT-I and SLT-II revealed that eventhough SLT-I and SLT-II have only an overall identity of 56%, the toxins share regions of high similarity (70-100%). Due to the degeneracy of the genetic code, twenty-seven percent of the base changes between SLT-I and SLT-II did not alter the corresponding amino acids, and in addition, twenty-nine percent of the amino acid changes conserved the type of amino acids substituted (i.e., hydrophobic, acidic or basic) (83). Sequence analyses revealed a relationship between the shiga toxin and SLT genes, and suggest an early diversion of SLT-I and SLT-II genes (83).

The family of shiga-like toxins also now includes related toxins produced by members of the genera Vibrio and some isolates of Salmonella typhimurium and Campylobacter jejuni which still have not been characterized in detail (127).

C. **Staphylococcus aureus enterotoxins**. The enterotoxins of Staphylococcus aureus form a group of five closely related proteins. These toxins have been designated A, B, C, D, and E, and are recognized as the causative agents of staphylococcal food poisoning (60). Ingestion of food contaminated with preformed enterotoxin leads to the rapid

development of the symptoms of vomiting and diarrhea that are characteristics of intoxication with these toxins (60, 84). In addition the staphylococcal enterotoxins have similar molecular weights (26,000 to 30,000), similar isoelectric points (7.0 to 8.6), and are relatively resistant to proteolytic enzymes and to heat (60). Another conserved feature among all of these toxins is the high content of the amino acids lysine, aspartic acid, and tyrosine. The existence of different serotypes of staphylococcal enterotoxins was established when Bergdoll et al. showed that antiserum to highly purified enterotoxin B did not neutralize the enterotoxins produced by a number of strains of S. aureus (19). However, there is evidence indicating that varying degrees of immunological relatedness exist between these toxins. The presence of common antigenic determinants between these enterotoxins has been demonstrated by immunodiffusion analysis, neutralization studies, and other sensitive immunoassays (24, 82, 97, 98, 167, 168, 182). In addition, significant nucleotide and amino acid sequence identity has been found between enterotoxins A to E (23, 25). This indicates that a common primary structure exists for all the enterotoxins, and further suggest that the shared immunobiological properties may result from these conserved regions.

D. **Botulinum and tetanus toxins**. Tetanus and botulinum toxins are potent neurotoxins produced by Clostridium tetani and Clostridium botulinum, respectively (22, 174).

The earliest manifestation of tetanus intoxication is muscle stiffness followed by spasm of the masseter muscles, also known as lock-jaw. As the disease progresses, tetanospasms cause clenching of the jaw, arching of the back, flexion of the arms, and extension of the lower extremities (22, 84). Death may occur during one of these spasms. All the symptoms in tetanus are attributable to a single serotype of the toxin (22).

Initial clinical manifestations of botulism intoxication include weakness, lassitude and dizziness. As the disease progresses, generalized weakness of muscle groups is observed, leading ultimately to respiratory paralysis and death (84, 174). Unlike tetanus toxin, there are seven serologically distinct botulinum toxins, which are designated A, B, C1, D, E, F, and G. Although no cross-neutralization is observed by heterologous antitoxin (with the exception of type E and F toxins), some antigenic similarities has been shown between these toxins (132, 133, 174, 184).

Although the clinical picture of poisoning by tetanus and botulinum toxins are quite different, there are several structural aspects of the toxins that point to more

similarities than differences between these toxins. Both toxins have molecular weights of approximately 150,000, and are synthesized as single polypeptide chains that are cleaved by proteases produced by the organism or by trypsin-like enzymes. The nicked toxins can be separated, following reduction of a disulfide bond, into two fragments designated heavy chain (Hc; MW, 100,000) and light chain (Lc; MW, 50,000) (22, 174). In addition, tetanus and botulinum toxins have been shown to bind to similar gangliosides (51, 157, 185). Both toxins are extremely specific for nerve cells, and part of this specificity is probably due to their ability to bind to unique receptors solely distributed in the nervous system. It has been suggested that tetanus and botulinum toxins have similar modes of action (117). These toxins seem to attach to nerve endings and impair neurotransmission by preventing the release of transmitter containing vesicles, however, the precise molecular mechanism leading to toxicity is not known.

Immunological cross-reactivity between tetanus and the botulinum toxins has been demonstrated by immunoassays with monoclonal and polyclonal antibodies (78, 184). Antibodies against synthetic peptides, representing different regions of tetanus toxin, recognized several of the botulinum toxins (78); this suggested that these toxins share regions of homology. In addition, the amino acid sequence of tetanus

toxin revealed areas of striking homologies with the partially determined sequence of some botulinum toxins (52).

Taken together, this information suggests that tetanus and botulinum neurotoxins are not only pharmacologically but also structurally related, and that they presumably stem from a common ancestral gene.

E. Clostridium perfringens iota toxin and Clostridium spiroforme iota-like toxin. Clostridium perfringens type E was implicated as a causative agent of enterotoxemia in domestic animals (28), and produces an iota toxin that presumably is involved in disease. Clostridium spiroforme has been identified as a cause of fatal enterotoxemia in rabbits (27, 34). Some strains of this organism produce an iota-like toxin that is immunologically related to the iota toxin produced by Clostridium perfringens (28, 169, 170, 171).

Both toxins are composed of two immunologically distinct polypeptide chains, designated i_a and i_b. No biological activity is shown by each individual component, however, when combined, an increase in biological activity is observed (170, 171). The toxins are lethal for mice and dermonecrotic for guinea pigs. The molecular weights for the components of iota and iota-like toxins are essentially identical: 48,000 and 67,000, and 45,000 and 68,000,

respectively. The isoelectric points also have been shown to be similar for the homologous components, and in addition, both toxins may require activation by proteases (169). The i_a component of both toxins has been shown to be an enzyme that ADP-ribosylates specific amino acids (158, 159). More recent studies have shown that nonmuscle actin acts as substrate for the incorporation of ADP-ribose (138). The i_b polypeptide may be a binding component that directs the toxin to target cells. The formation of hybrid iota toxins, using complementary components between both species has been achieved (169), demonstrating that even though the toxins are not identical, enough compatibility still remains to allow for hybrid toxin activity.

The production of an i_a-like protein by C. difficile has been reported recently (139). The purified protein has a molecular weight of 43,000 daltons, an isoelectric point of 7.8 and is unrelated to the toxins A and B produced by this organism. This protein, designated CDT, shows no cytotoxic or lethal activities, and like i_a and i_a-like, catalyzes the transfer of an ADP-ribose from NAD to cell actin. Monospecific antibodies to CDT were shown to react with a 43,000 protein in the culture filtrate of C. perfringens type E, which suggested that this enzyme shares antigenic determinants with i_a (139). Immunological cross-reactivity was further demonstrated when both, i_a and i_a-like toxins

were isolated by immunoaffinity chromatography using antibodies to CDT (138). It would be interesting to determine if CDT can complement with C. perfringens i_b or with i_b-like of C. spiroforme, to give expression of toxic activity.

LITERATURE CITED

1. **Abrams, G.D. , M. Allo, G.D. Rifkin, R. Fekety, and J. Silva, Jr.** 1980. Mucosal damage mediated by clostridial toxin in experimental clindamycin-associated colitis. Gut 21:493-499.

2. **Allo, M., J. Silva, and R. Fekety.** 1979. Prevention of clindamycin-induced colitis in hamsters by Clostridium sordellii antitoxin. Gastroenterology 76:351-355.

3. **Allo, M.** 1980. Clostridial toxins in strangulation obstruction and antibiotic-related colitis. J. Surg. Res. 28:421-425.

4. **Al Mashat, R.R., and D.J. Taylor.** 1983. Bacteria in enteric lesions of cattle. Vet. Rec. 112:5-10.

5. **Al Mashat, R.R., and D.J. Taylor.** 1983. Clostridium sordellii in enteritis in an adult sheep. Vet. Rec. 112:19.

6. **Al Mashat, R.R., and D.J. Taylor.** 1983. Production of diarrhoea and enteric lesions in calves by the oral inoculation of pure cultures of Clostridium sordellii. Vet. Rec. 112:141-146.

7. **Altemeier, W.A., R.P. Hummel, and E.O. Hill.** 1963. Staphylococcal enterocolitis following antibiotic theraphy. Ann. Surg. 157:847.

8. **Arseculeratne, S.N., R.G. Panabokke, and S. Wijensundera.** 1969. The toxins responsible for the lesions of Clostridium sordellii gas gangrene. J. Med. Microbiol. 2:37-53.

9. **Aswell, J.E., M. Ehrich, R. Van Tassell, G.C. Tsai, L.V. Holdeman, and T.D. Wilkins.** 1979. Characterization and comparison of Clostridium difficile and other clostridial toxins. In D. Schlessinger (ed.) Microbiology-1979 American Society for Microbiology, p. 272-275, Washington, D.C.

10. **Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Ueno, and Y. Nozawa.**
1984. Biochemical characterization and biologic actions of two toxins (D-1 and D-2) from Clostridium difficile. Rev. Infect. Dis. 6:S11-S20.

11. **Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nazawa.** 1981. Two toxins (D-1 and D-2) of Clostridium difficile causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. 2:629-

635.

12. **Bartlett, J.G.** 1979. Antibiotic-associated pseudomembranous colitis. Rev. Infect. Dis. 1:530-539.

13. **Bartlett, J.G.** 1981. Antimicrobial agents implicated in Clostridium difficile toxin-associated diarrhea or colitis. John Hopkins Med. J. 149:6-9.

14. **Bartlett, J.G., T.-W. Chang, M. Gurwith, S.L. Gorbach, and A.B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin producing clostridia. New Eng. J. Med. 298:531-534.

15. **Bartlett, J.G., T.-W. Chang, N. Moon, and A.B. Onderdonk.** 1978. Antibiotic-induced lethal enterocolitis in hamsters:studies with eleven agents and evidence to support the pathogenic role of of toxin producing clostria. Am. J. Vet. Res. 39:1525-1530.

16. **Bartlett, J.G., N. Moon, T.-W. Chang, N. Taylor and A.B. Onderdonk.** 1978. Role of Clostridium difficile in antibiotic-associated pseudomembranous colitis. Gastroenterology 75:778-782.

17. **Bartlett, J.G., A.B. Onderdonk, R. Cisneros, and D.L. Kasper.** 1977. Clindamycin-associated colitis due to toxin-producing species of Clostridium in hamsters. J. Infect. Dis. 136:701-705.

18. **Bartlett, J.G., N.S. Taylor, T.-W. Chang, and J. Dzink.** 1980. Clinical and laboratory observations in Clostridium difficile colitis. Am. J. Clin. Nutr. 33:2521-2526.

19. **Bergdoll, M.S., M.J. Surgalla, and M.J. Dack.** 1959. Staphyloccal enterotoxin. Identification of a precipitating antibody with enterotoxin-neutralizing property. J. Immun. 83:334-338.

20. **Bernheimer, A.W.** 1976. Sulfhydryl-activated toxins, p.85-97. In A.W. Bernheimer (ed.), Mechanisms in bacterial toxinology. John Wiley and Sons, New York.

21. **Betley, M.J., and J.J. Mekalanos.** 1988. Nucleotide sequence of the type A staphylococcal enterotoxin gene. J. Bact. 170:34-41.

22. **Bizzini, B.** 1979. Tetanus toxin. Microbiol. Rev. 43:224-240.

23. **Bohach, G.A., and P.M. Schlievert.** 1987. Nucleotide sequence of the staphylococcal enterotoxin C1 gene and relatedness to other pyrogenic toxins. Mol. Gen. Genet. 209:15-20.

24. **Bohach, G.A., C.J. Hovde, J.P. Handley, and P.M. Schlievert.** 1988. Cross-neutralization of staphylococcal and streptococcal pyrogenic toxins by monoclonal and polyclonal antibodies. Infect. Immun. 56:400-404.

25. **Bohach, G.A., and P.M. Schlievert.** 1989. Conservation of the biologically active portions of staphylococcal enterotoxins C1 and C2. Infect. Immun. 57:2249-2252.

26. **Bornside, R.H., C.E. Floyd, and I. Cohn.** 1964. Clostridium sordellii in strangulation obstruction: further studies of serial changes of intestinal contents. J. Surg. Res. 4:233-239.

27. **Borriello, S.P., and R.J. Carman.** 1983. Association of iota-like toxin of Clostridium spiroforme with both spontaneous and antibiotic-associated diarrhea and colitis in rabbits. J. Clin. Microbiol. 17:414-418.

28. **Borriello, S.P, and R.J. Carman.** 1985. Clostridial diseases of the gastrointestinal tract in animals, p.195-222. In S.P. Borriello (ed.), Clostridia in gastrintestinal disease, CRC Press, Inc., Boca Raton, Florida.

29. **Borriello, S.P., and P. Honour.** 1981. Simplified procedure for the routine isolation of Clostridium difficile from faeces. J. Clin. Pathol. 34:1124-1127.

30. **Borriello, S.P., and H.E. Larson.** 1981. Antibiotics and pseudomembranous colitis. J. Antimicrob. Chemother. 7:A53-62.

31. **Borriello, S.P.,and H.E. Larson.** 1985. Pseudomembranous and antibiotic-associated colitis, p.145-164. In S.P. Borriello (ed.), Clostridia in gastrointestinal disease, CRC Press, Inc., Boca Raton, Florida.

32. **Brooks, M.E., and M. Sterne.** 1956. Occurrence of Clostridium sordellii in Great Britain. Vet. Rec. 68:121-122.

33. **Burdon, D.W., R.H. George, G.A.G. Mogg, Y. Arabi, H. Thompson, M. Johnson, J. Alexander-Williams, and M.R.B. Keighley.** 1981. Faecal toxin and severity of antibiotic-

associated pseudomembranous colitis. J. Clin. Pathol. 34:548-551.

34. **Carman, R.J., and S.P. Borriello.** 1982. Clostridium spiroforme isolated from rabbits with diarrhoea. Vet. Rec. 111:461-462.

35. **Carroll, S.F., and R.J. Collier.** 1988. Amino acid sequence homology between the enzymic domains of diphtheria toxin and Pseudomonas aeruginosa exotoxin A. Mol. Microbiol. 2:293-296.

36. **Chang, T.-W., J.G. Bartlett, S.L. Gorbach, and A.B. Onderdonk.** 1978. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis. Infect. Immun. 20:526-529.

37. **Chang, T.-W., S.L. Gorbach, and J.G. Bartlett.** 1978. Neutralization of Clostridium difficile toxin by Clostridium sordellii antitoxins. Infect. Immun. 22:418-422.

38. **Chang, T.-W., M. Lauerman, and J.G. Bartlett.** 1979. Cytotoxicity assay in antibiotic-associated colitis. J. Infect. Dis. 140:765-770.

39. **Clements, J.D., and R.A. Finkelstein.** 1978. Immunological cross-reactivity between heat-labile enterotoxin(s) of Escherichia coli and subunits of Vibrio cholerae enterotoxin. Infect. Immun. 21:1036-1039.

40. **Clements, J.D., and R.A. Finkelstein.** 1978. Demonstration of shared and unique immunological determinants in enterotoxins from Vibrio cholerae and Escherichia coli. Infect. Immun. 22:709-713.

41. **Cohen, L.E., C.J. McNeill, and R.F. Wells.** 1973. Clindamycin-associated colitis. J. Am. Med. Assoc. 223:1379-1380.

42. **Davis, J.S.** 1974. Severe colitis following lincomycin and clindamycin therapy. Am. J. Gastroenterol. 62:16-23.

43. **Dearing, W.H., A.H. Baggenstoss, and L.A. Weed.** 1960. Studies on the relationship of Staphylococcus aureus to pseudomembranous enteritis and to postantibiotic enteritis. Gastroenterology 38: 441-451.

44. **Donta, S.T.** 1977. The risk of diarrhea and colitis with antibiotic therapy. Geriatrics 32:103-106.

45. **Donta, S.T., G.M. Lamps, R.W. Summers, and T.D. Wilkins.** 1980. Cephalosporin-associated colitis and Clostridium difficile. Arch. Intern. Med. 140:574-576.

46. **Donta, S.T., and S.J. Shaffer.** 1980. Effects of Clostridium difficile toxin on tissue-cultured cells. J. Infect. Dis. 141:218-222.

47. **Donta, S.T., N.M. Sullivan, and T.D. Wilkins.** 1982. Differential effects of Clostridium difficile toxins on tissue-cultured cells. J. Clin. Microbiol. 15:1157-1158.

48. **Dorner, F., H. Jaksche, and W. Stockl.** 1976. Escherichia coli enterotoxin: purification, partial characterization, and immunological observations. J. Infect. Dis. 133:S142-S156.

49. **Dupont, H.L.** 1985. Shigella species (bacillary dysentery), p.1269-1274. In G.L. Mandell, R.G. Douglas, Jr., and J.E. Benett (ed.), Principles and practice of infectious diseases, 2nd ed. John Wiley & Sons, Inc., New York.

50. **Ehrich, M., R.L. VanTassell, J.M. Libby, and T.D. Wilkins.** 1980. Production of Clostridium difficile antitoxin. Infect. Immun. 28:1041-1043.

51. **Eidels, L., R.L. Proia, and D.A. Hart.** 1983. Membrane receptors for bacterial toxins. Microbiol. Rev. 47:596-620.

52. **Eisel, U., W. Jarausch, K. Goretzki, A. Henschen, J. Engels, U. Weller, M. Hudel, E. Habermann, and H. Niemann.** 1986. Tetanus toxin: primary structure, expression in E. coli, and homology with botulinum toxins. EMBO J. 5:2495-2502.

53. **Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and Igarashi.** 1988. The site of action of verotoxin (VT2) from Escherichia coli 0157:H7 and of shiga toxin on eukaryotic ribosomes: RNA N-glycosidase activity of the toxins. Euro. J. Biochem. 171:45-50.

54. **Evans, D.G., D.J. Evans, Jr., and S.L. Gorbach.** 1973. Identification of enterotoxigenic Escherichia coli and serum antitoxin activity by the vascular permeability factor assay. Infect. Immun. 8:731-735.

55. **Fekety, R., J. Silva, R.A. Browne, G.D. Rifkin and J.R. Ebright.** 1979. Clindamycin-induced colitis. Am. J. Clin. Nutr. 32:244-250.

56. **Fernie, D.S., R.O. Thompson, I. Batty, and P.D. Walker.** 1982. Active and passive immunization to protect against antibiotic-associated caecitis in hamsters. Develop. Biol. Stand. 53:325-332.

57. **Finkelstein, R.A., M.F. Burks, A. Zupan, W.S. Dallas, C.O. Jacob, and D.S. Ludwig.** 1987. Epitopes of the cholera family of enterotoxins. Rev. Infect. Dis. 9:544-561.

58. **Finkelstein, R.A., M.F. Burks, A. Zupan, W.S. Dallas, C.O. Jacob, and D.S. Ludwig.** 1987. Antigenic determinants of the cholera/coli family of enterotoxins. Rev. Infect. Dis. 9:S490-S502.

59. **Finkelstein, D.S., and F. Dorner.** 1985. Cholera enterotoxin (choleragen). Pharmac. Ther. 27:37-47.

60. **Freer, J.H., and J.P. Arbuthnott.** 1986. Toxins of Staphylococcus aureus, p. 616-622. In F. Dorner And J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon press., New York.

61. **George, W.L., and S.M. Finegold.** 1985. Clostridia in the human gastrointestinal flora, p.1-38. In S.P. Borriello (ed.), Clostridia in gastrointestinal disease, CRC Press, Inc., Boca Raton, Florida.

62. **George, W.L., R.D. Rolfe, and S.M. Finegold.** 1982. Clostridium difficile and its cytotoxin in feces of patients with antimicrobial agent-associated diarrhea and miscellaneous conditions. J. Clin. Microbiol. 15:1049-1053.

63. **George, W,L., V.L. Sutter, D. Citron, and S.M. Finegold.** 1979. Selective and differential medium for isolation of Clostridium difficile. J. Clin. Microbiol. 9:214-219.

64. **George, W.L., V.L. Sutter, and S.M. Finegold.** 1977. Antimicrobial agent-induced diarrhea - a bacterial disease. J. Infect. Dis. 136:822-828.

65. **George, W.L., V.L. Sutter, E.C. Goldstein, S.L. Ludwig, and S.M. Finegold.** 1978. Aetiology of antimicrobial-agent-associated colitis. Lancet i:802-803.

66. **George, R.H., J.M. Symonds, F. Dimock, J.D. Brown, Y. Arabi, N. Shinagawa, M.R.B. Keighley, J. Alexander-Williams, and D.W. Burdon.** 1978. Identification of Clostridium difficile as a cause of pseudomembranous colitis. Br. Med. J. 1:695.

67. **Gill, D.M., and R. Meren.** 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. 75:3050-3054.

68. **Gill, D.M., and S.H. Richardson.** 1980. Adenosine diphosphate-ribosylation of adenylate cyclase catalysed by heat labile enterotoxin of Escherichia coli: comparison with cholera toxin. J. Infect. Dis. 141:64-70.

69. **Gorbach, S.L., and J.G. Bartlett.** 1977. Pseudomembranous enterocolitis: a review of its diverse forms. J. Infect. Dis. 135:589-594.

70. **Gorbach, S.L., and H. Thadepalli.** 1975. Isolation of Clostridium in human infections: evaluation of 114 cases. J. Infect. Dis. 131:S81-85

71. **Greenberg, R.N., and R.L. Guerrant.** 1986. E. coli heat-stable enterotoxin, p. 115-136. In F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, New York.

72. **Guillaumie, M., A. Pesce de Fagonde, and A. Kreguer.** 1950. Hemolysine oxydable de Clostridium bifermentans. Ann. Inst. Pasteur (Paris) 79:20-32.

73. **Gurwith, M.J., H.R. Rabin, K. Love, and the cooperative Antibiotic Diarrhea Study Group.** 1977. Diarrhea associated with clindamycin and ampicillin therapy: preliminary results of a cooperative study. J. Infect. Dis. 135:S104-S110.

74. **Gyles, C.L.** 1974. Relationships among heat-labile enterotoxins of Escherichia coli and Vibrio cholerae. J. Infect. Dis. 129:277-283.

75. **Hall, I.C.** 1929. The occurrence of Bacillus sordellii in icterohemoglobinuria of cattle in Nevada. J. Infect. Dis. 45:156-162.

76. **Hall, I.C., and S.H. Gray.** 1931. Septic peritonitis in man due to Bacillus sordellii. Arch. Path. 12:245-252.

77. **Hall, I.C., and J.M. Scott.** 1927. Bacillus sordellii, a cause of malignant edema in man. J. Infect. Dis. 41:329-335.

78. **Halpern J.L., L.A. Smith, K.B. Seamon, K.A. Groover, and W.H. Habig.** 1989. Sequence homology between tetanus and botulinum toxins detected by antipeptide antibody. Infect.

Immun. 57:18-22.

79. **Hibb, C.M., D.R. Johnson, K. Reynolds, and R. Harrington, Jr.** 1977. Clostridium sordellii isolated from foals. Vet.Med./SAC 72:256-258.

80. **Hogan, S.F., and K. Ireland.** 1989. Fatal acute spontaneous endometritis resulting from Clostridium sordellii. Am. J. Clin. Pathol. 91:104-106.

81. **Humphrey, C.D., J.C. Pittman, and F.E. Pittman.** 1975. A hamster model for lincomycin colitis. Clin. Res. 23:27A.

82. **Hynes, W.L., C.R. Weeks, J.J. Iandolo, and J.J. Ferretti.** 1987. Immunologic cross-reactivity of type A streptococcal exotoxin (erythrogenic toxin) and staphylococcal enterotoxins B and C1. Infect. Immun. 55:837-838.

83. **Jackson, M.P., R.J. Neill, A.D. O'Brien, R.K. Holmes, and J.W. Newland.** 1987. Nucleotide sequence analysis and comparison of the structural genes for shiga-like toxin I and shiga-like toxin II encoded by bacteriophages from Escherichia coli 933. FEMS Microbiol. Lett. 44:109-114.

84. **Joklik, W.K., H.P. Willet, and D.B. Amos (ed.).** 1984. Zinsser microbiology. Appleton-Century-Crofts, Norwalk, Conn.

85. **Jones, C.L., and S.A. Khan.** 1986. Nucleotide sequence of the enterotoxin B gene from Staphylococcus aureus. 1986. J. Bact. 166:29-33.

86. **Keusch, G.T., A. Donohue-Rolfe, and M. Jacewicz.** 1985. Shigella toxin and the pathogenesis of shigellosis, p.193-214. In D.Evered And J. Whelan (ed.), Microbial toxins and diarrhoeal disease. Ciba Foundation Symposium 112. Pittman Publishers, London.

87. **Khan, M.Y., and W.H. Hall.** 1966. Staphylococcal enterocolitis - treatment with oral vancomycin. Ann. Int. Med. 65:1-8.

88. **Kim, P.-H., and R.D. Rolfe.** 1987. The protective role of antibody to toxin A in Clostridium difficile-induced ileocecitis. Abstr. Annu. Meet. Am. Soc. Microbiol, B-222, p. 62.

89. **Klipstein, F.A., and R.F. Engert.** 1977. Immmunological

interrelationships between cholera toxin and heat-labile and heat-stable enterotoxins of coliform bacteria. Infect. Immun. 18:110-117.

90. **Kramer, I.R.H.** 1948. Fatal staphylococcal enteritis developing during streptomycin therapy by mouth. Lancet 255:646.

91. **Krivan, H.C., G.F. Clark, D.F. Smith, and T.D. Wilkins.** 1986. Cell surface binding site for Clostridium difficile enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. Infect. Immun. 53:573-581.

92. **Krivan, H.C., and T.D. Wilkins.** 1987. Purification of Clostridium difficile toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. 55:1873-1877.

93. **Larson, H.E., J.V. Parry, A.B. Price, D.R. Davies , J. Dolby, and D.A.J. Tyrrell.** 1977. Undescribed toxin in pseudomembranous colitis. Br. Med. J. 1:1246-1248.

94. **Larson, H.E., and A.B. Price.** 1977. Pseudomembranous colitis: presence of clostridial toxin . Lancet ii:1312-1314.

95. **Larson, H.E., A.B. Price, P. Honour, and S.P. Borrielo.** 1978. Clostridium difficile and the aetiology of pseudomembranous colitis. Lancet i:1063-1066.

96. **Laughon, B.E., R.P. Viscidi, S.L. Gdovin, R.H. Yolken, and J.G. Bartlett.** 1984. Enzyme immunoassays for detection of Clostridium difficile toxins A and B in fecal specimens. J. Infect. Dis. 149:781-788.

97. **Lee, A.C.-M., R.N. Robbins, and M.S. Bergdoll.** 1978. Isolation of specific and common antibodies to staphylococcal enterotoxins A and E by affinity-chromatography. Infect. Immun. 21:387-391.

98. **Lee, A.C.-M., R.N. Robbins, R.F. Reiser, and M.S. Bergdoll.** 1980. Isolation of specific and common antibodies to staphylococcal enterotoxins B, C1, and C2. Infect. Immun. 27:431-434.

99. **Lewis, G.M., and M.G. Macfarlane.** 1953. The lecithinase of Clostridium bifermentans toxin. Biochem. J. 54:138-142.

100. **Libby, J.M., B.S. Jortner, and T.D. Wilkins.** 1982.

Effects of two toxins of Clostridium difficile in antibiotic-associated cecitis in hamsters. Infect. Immun. 36:822-829.

101. Lillie, R.D. 1938. Pathologic histology in mice produced by intravenous inoculation with the toxin of Clostridium sordellii (bifermentans). Publ. Hlth. Rep. Wash. 53:113-121.

102. Lima, A.A.M., D.M. Lyerly, T.D. Wilkins, D.J. Innes, and R.L. Guerrant. 1988. Effects of Clostridium difficile toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. Infect. Immun. 56:582-588.

103. Lusk, R.H., F.R. Fekety, Jr.., J.Silva, Jr., T. Bodendorfer, B.J. Devine, H. Kawanishi, L.Korff, D. Nakauchi, S. Rogers, and S.B. Siskin. 1977. Gastrointestinal side effects of clindamycin and ampicillin therapy. J. Infect. Dis. 135:S111-S119.

104. Lusk, R.H., R. Fekety, J. Silva, R.A. Browne, D.H. Ringler, and G.D. Abrams. 1978. Clindamycin-induced enterocolitis in hamsters. J. Infect. Dis. 137:464-475.

105. Lyerly, D.M., H.C. Krivan, and T.D. Wilkins. 1988. Clostridium difficile: its disease and toxin. Clin. Microbiol. Rev. 1:1-18.

106. Lyerly, D.M., D.E. Lockwood, S.J. Richardson, and T.D. Wilkins. 1982. Biological activities of toxins A and B of Clostridium difficile. Infect. Immun. 35:1147-1150.

107. Lyerly, D.M., C.J. Phelps, J. Toth, and T.D. Wilkins. 1986. Characterization of toxins A and B of Clostridium difficile with monoclonal antibodies. Infect. Immun. 54:70-76.

108. Lyerly, D.M., M.D. Roberts, C.J. Phelps, and T.D. Wilkins. 1986. Purification and properties of toxins A and B of Clostridium difficile. FEMS Microbiol. Lett. 33:31-35.

109. Lyerly, D.M., K.E. Saum, D.K. MacDonald, and T.D. Wilkins. 1985. Effects of Clostridium difficile toxins given intragastrically to animals. Infect. Immun. 47:349-352.

110. MacGregor, J.A., G.L. Lovell, and D. Soper. 1988. Clostridium sordellii mediated toxic shock and myonecrosis. Abstr. Ann. Meet. Am. Soc. Microbiol. , p. 78.

111. **MacLennan, J.D.** 1962. The histotoxic clostridial infections of man. Bact. Rev. 26:177-276

112. **Meador III, J., and R.K. Tweten.** 1988. Purification and characterization of toxin B from Clostridium difficile. Infect. Immun. 56:1708-1714.

113. **Medline, A., D.H. Shin, and N.M. Medline.** 1976. Pseudomembranous colitis associated with antibiotics. Human Pathol. 7:693-703.

114. **Meleney, F.L., F.B. Humphreys, and L. Carp.** 1927. An unusual fatal operative wound infection yielding a pathogenic anaerobe of the gas gangrene group not hitherto described. Surg. Gynec. Obstet. 45:775-778.

115. **Meleney, F.L., F.B. Humphreys, and L. Carp.** 1928. Pathogenic anaerobic bacillus not hitherto described cultured from fatal operative wound infection. Proc. Soc. Exp. Biol. 24:675-677.

116. **Mellanby, J., and A.B. White.** 1968. The independence of the lysolecithinase activity of extracts of Clostridium sordellii from their lethal, edema-producing and hemorrhagic actions. J. Gen. Microbiol. 52:455-459.

117. **Mellanby, J.** 1984. Comparative activities of tetanus and botulinum toxins. J.Neurosci. 11:29-34.

118. **Middlebrook, J.L., and R.B. Dorland.** 1984. Bacterial toxins: cellular mechanisms of action. Microbiol. Rev. 48:199-221.

119. **Miles, E.M., and A.A. Miles.** 1947. The lecithinase of Clostridium bifermentans and its relation to the alpha-toxin of Clostridium welchii. J. Gen. Microbiol. 1:385-399.

120. **Miles, E.M., and A.A. Miles.** 1950. The relation of toxicity and enzyme activity in the lecithinases of Clostridium bifermentans and Clostridium welchii. J. Gen. Microbiol. 4:22-35.

121. **Mitchell, T.J., J.M. Ketley, S.C. Haslam, J. Stephen, D.W. Burdon, D.C.A. Candy, and R. Daniel.** 1986. The effect of toxin A and B of Clostridium difficile on rabbit ileum and colon. Gut 27:78-85.

122. **Moore, W.E.C., E.P. Cato, and L.V. Holdeman.** 1969. Anaerobic bacteria of the gastrointestinal flora and their

occurrence in clinical infections. J. Infect. Dis. 119:641-649.

123. **Nakamura, S., H. Ogura, J. Tanaka, N. Tanabe, K. Hamakawa, M. Hatano, and S. Nishida.** 1984. Difference in susceptibility of various cell cultures to cytotoxic culture filtrates of Clostridium sordellii. Microbiol. Immunol. 28:493-497.

124. **Nakamura, S., N. Tanabe, K. Yamakawa, and S. Nishida.** 1983. Cytotoxin production by Clostridium sordellii strains. Microbiol. Immunol. 27:495-502.

125. **Newland, J.W., N.A. Strockbine, and R.G. Neill.** 1987. Cloning of genes for production of Escherichia coli shiga-like toxin type II. Infect. Immun. 55:2675-2680.

126. **Nord, C.E., T. Wadstrom, K. Dornbusch, and B. Wretlind.** 1975. Extracellular proteins in five clostridial species from human infections. Med. Microbiol. Immunol. 161:145-154.

127. **O'Brien, A.D., and R.K. Holmes.** 1987. Shiga and shiga-like toxins. Microbiol. Rev. 51:206-220

128. **O'Brien, A.D., and G.D. LaVeck.** 1982. Immunochemical and cytotoxic activities of Shigella dysenteriae 1 (shiga) and shiga-like toxins. Infect. Immun. 35:1151-1154.

129. **O'Brien, A.D., and G.D. LaVeck.** 1983. Purification and characterization of a Shigella dysenteriae 1-like toxin produced by Escherichia coli. Infect. Immun. 40:675-683.

130. **O'Brien, A.D., G.D. LaVeck, M.R. Thompson, and S.B. Formal.** 1982. Production of Shigella dysenteriae type 1-like cytotoxin by Escherichia coli. J. Infect. Dis. 146:763-769.

131. **O'Brien, A.D., J.W. Newland, S.F. Miller, R.K. Holmes, H. Williams Smith, and S.B. Formal.** 1984. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. Science 226:694-696.

132. **Oguma, K., S. Murayama, B. Syuto, H. Iida, and S. Kubo.** 1984. Analysis of antigenicity of Clostridium botulinum type C1 and D toxins by polyclonal and monoclonal antibodies. Infect. Immun. 43:584-588.

133. **Oguma, K., B. Syuto, H. Iida, and S. Kubo.** 1980. Antigenic similarity of toxins produced by Clostridium

botulinum type C and D strains. Infect. Immun. 30:656-660.

134. **Olsnes, S., R. Reisbig, and K. Eiklid.** 1981. Subunit structure of Shigella cytotoxin. J. Biol. Chem. 256:8732-8738.

135. **Pittman, F.E., J.C. Pittman, and C.D. Humphrey.** 1974. Colitis following oral lincomycin therapy. Arch. Intern. Med. 134:368-372.

136. **Popoff, M.R.** 1984. Bacteriological examination in enterotoxaemia of sheep and lamb. Vet. Rec. 114:324.

137. **Popoff, M.R.** 1987. Purification and characterization of Clostridium sordellii lethal toxin and cross-reactivity with Clostridium difficile cytotoxin. Infect. Immun. 55:35-43.

138. **Popoff, M.R., and P. Boquet.** 1988. Clostridium spiroforme toxin is a binary toxin which ADP-ribosylates cellular actin. Biochem. Biophys. Res. Commun. 152:1361-1368.

139. **Popoff, M.R., E.J. Rubin, D.M. Gill, and P. Boquet.** 1988. Actin-specific ADP-ribosyltransferase produced by a Clostridium difficile strain. Infect. Immun. 56:2299-2306.

140. **Pothoulakis, C., L.M. Barone, R. Ely, B. Faris, M.E. Clark, C. Franzblau, and J.T. LaMont.** 1986. Purification and properties of Clostridium diffile toxin B. J. Biol. Chem. 261:1316-1321.

141. **Prevot, A.R., and A.J. Malgras.** Recherches sur l'hemolysine de Clostridium sordellii. Ann. Inst. Pasteur 78:133-135

142. **Price, A.B., and D.R. Davies.** 1977. Pseudomembranous colitis. J. Clin. Path. 30:1-12.

143. **Price, A.B., H.E. Larson, and J. Crow.** 1979. Morphology of experimental antibiotic-associated enterocolitis in the hamster: a model for human pseudomembranous colitis and antibiotic associated diarrhea. Gut 20:467-475.

144. **Rehg, J.** 1980. Cecal toxin(s) from guinea pigs with clindamycin-associated colitis, neutralized by Clostridrium sordellii antitoxin. Infect. Immun. 27:387-390.

145. **Reiner, L., M.J. Schlesinger, and G.M. Miller.** 1952.

Pseudomembranous colitis following aureomycin and chloramphenicol. Arch. Pathol. 54:39-67.

146. Richards, K.L., and S.D. Douglas. 1978. Pathophysiologycal effects of Vibrio cholerae and enterotoxigenic Escherichia coli and their exotoxins on eucaryotic cells. Microbiol. Rev. 42:592-613.

147. Richards, S.M. 1982. Clostridium sordellii in lambs. Vet. Rec. 111:22.

148. Rifkin, G.D., F.R. Fekety, and J, Silva. 1978. Neutralization by Clostridium sordellii antitoxin of toxins implicated in clindamycin-induced cecitis in the hamster. Gastroenterology 75:422-424.

149. Rifkin, G.D., F.R. Fekety, J. Silva, Jr., and R.B. Sack. 1977. Antibiotic-induced colitis. Implication of a toxin neutralized by Clostridium sordellii antitoxin. Lancet ii:1103-1106.

150. Rifkin, G.D., J. Silva, Jr., and R. Fekety. 1978. Gastrointestinal and systemic toxicity of fecal extracts from hamsters with clindamycin-induced colitis. Gastroenterology. 74:52-57.

151. Rihn, B., J.M. Scheftel, R.Girardot, and H. Monteil. 1984. A new purification procedure for Clostridium difficile enterotoxin. Biochem. Biophys. Res. Commun. 124:690-695.

152. Robertson , D. C., J.L. McDonel, and F. Dorner. E. coli heat-labile enterotoxin, p. 77-114. In F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, New York.

153. Roggentin, P., B. Wigbert, and R. Schauer. 1987. Purification and characterization of sialidase from Clostridium sordellii G12. Glycoconjugate J. 4:349-359.

154. Sadoff, J.C., G.A. Buck, B.H. Iglewski, M.J. Bjorn, and N.B. Groman. 1982. Immunological cross-reactivity in the absence of DNA homology between Pseudomonas toxin A and diphtheria toxin. Infect. Immun. 37:250-254.

155. Saissac, R., and M. Raynaud. 1951. Extraction de la toxine de Clostridium sordellii a partir des corps microbiens. Ann. Inst. Pasteur (Paris) 80:431-435.

156. Saxena, S.K., A.D. O'Brien and E.J. Ackerman. 1989.

Shiga toxin, shiga-like toxin II variant, and ricin are all single-site RNA glycosidases of 28S RNA when microinjected into Xenopus oocytes. J. Biol. Chem. 264:596-601.

157. **Simpson, L.L., and M.M. Rapport.** 1971. The binding of botulinum toxin to membrane lipids: sphingolipids, steroids, and fatty acids. J. Neurochem. 18:1751-1759.

158. **Simpson, L.L., B.G. Stiles, H.H. Zepeda, and T.D. Wilkins.** 1987. Molecular basis for the pathological actions of Clostridium perfringens iota toxin. Infect. Immun. 55:118-122.

159. **Simpson, L.L., B.G. Stiles, H. Zepeda, and T.D. Wilkins.** 1989. Production by Clostridium spiroforme of an iotalike toxin that possesses mono(ADP-ribosyl)transferase activity: identification of a novel class of ADP-rybosyltransferases. Infect. Immun. 57:255-261.

160. **Small, J.D.** 1968. Fatal enterocolitis in hamsters given lincomycin hydrochloride. Lab. Anim. Care 18:411-420.

161. **Smith, L.DS., J.W. Safford, and W.W. Hawkins.** 1962. Clostridium sordellii infection in sheep. Cornell Vet. 52:62-68.

162. **Smith, L.DS., and B.L. Williams.** 1984. The Pathogenic Anaerobic Bacteria.(3rd ed.), Charles C. Thomas. Springfield, Illinois.

163. **Soper, D.E.** 1986. Clostridial myonecrosis arising from an episiotomy. Obstet. Gynecol. 68:26S-28S.

164. **Sordelli, A.** 1922. Un germen anaerobio de las gangrenas gaseosas. Rev. Asoc. Med. Argent. 35:217-221.

165. **Sordelli, A.** 1922. Sobre suero antigangrenoso. Rev. Asoc. Med. Argent. 35:308-310.

166. **Speare, G.S.** 1954. Staphylococcal pseudomembranous enterocolitis, a complication of antibiotic therapy. Amer. J. Surg. 88:523.

167. **Spero, L., and B.A. Morlock.** 1979. Cross-reactions between tryptic polypeptides of staphylococcal enterotoxins B and C. J. Immunol. 122:1285-1289.

168. **Spero, L., B.A. Morlock, and J.F. Metzger.** 1978. On the cross-reactivity of staphylococcal enterotoxins, A, B, and

C. J. Immunol. 120:86-89.

169. **Stiles, B.G.** 1987. Purification and characterization of Clostridium perfringens iota toxin. Ph.D. Thesis. Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University.

170. **Stiles, B.G., and T.D. Wilkins.** 1986. Clostridium perfringens iota toxin: synergism between two proteins. Toxicon 24:767-773.

171. **Stiles B.G., and T.D. Wilkins.** 1986. Purification and characterization of Clostridium perfringens iota toxin. Dependence on two nonlinked proteins for biological activity. Infect. Immun. 54:683-688.

172. **Strockbine, N.A., M.P. Jackson, L.M. Sung, R.K. Holmes, and A.D. O'Brien.** 1988. Cloning and sequencing of the genes for shiga toxin from Shigella dysenteriae type 1. J. Bact. 170:1116-1122.

173. **Strockbine, N.A., L.R.M. Marques, J.W. Newland, H. Williams Smith, R.K. Holmes, and A.D. O'Brien.** 1986. Two toxin-converting phages from Escherichia coli 0157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect. Immun. 53:135-140.

174. **Sugiyama, H.** Clostridium botulinum neurotoxin. 1980. Microbiol. Rev. 44:419-448.

175. **Sullivan, N.M., D.M. Lyerly, and T.D. Wilkins.** 1983. Purification of Clostridium difficile toxin B and demonstration of multiple forms of the toxin. Abstr. Ann. Meet. Am. Soc. Microbiol., p. 26.

176. **Sullivan, N.M., S. Pellett, and T.D. Wilkins.** 1982. Purification and characterization of toxins A and B of Clostridium difficile. Infect. Immun. 35:1032-1040.

177. **Taylor, N.S., G.M. Thorne, and J.G. Bartlett.** 1980. Separation of an enterotoxin from the cytotoxin of Clostridium difficile. Clin. Res. 28:285A.

178. **Taylor, N.S., G.M. Thorne, and J.G. Bartlett.** 1981. Comparison of two toxins produced by Clostridium difficile. Infect. Immun. 34:1036-1043.

179. **Tedesco, F.J.** 1984. Antibiotics associated with Clostridium difficile mediated diarrhea and/or colitis, p.

3-8. In S.P. Borriello (ed.), Antibiotic associated diarrhea and colitis: Clostridium difficile in gastrointestinal disorders, Martinus Nijhoff Publishers, Boston.

180. **Thelestam, M., and M. Bronnegard.** 1980. Interaction of cytopathogenic toxin from Clostridium difficile with cells in tissue culture. Scand. J. Infec. Dis. Suppl. 22:16-29.

181. **Thelestam, M., and I, Florin.** 1984. Interaction of Clostridium difficile toxin B (cytotoxin) with cultured cells, p.103-118. In S.P. Boriello (ed.), Antibiotic associated diarrhoea and colitis: Clostridium difficile in gastrointestinal disorders, Martinus Nijhoff Publishers, Boston.

182. **Thompson, N.E., M.J. Ketterhagen, and M.S. Bergdoll.** 1984. Monoclonal antibodies to staphylococcal enterotoxins B and C: cross-reactivity and localization of epitopes on tryptic fragments. Infect. Immun. 45:281-285.

183. **Torres, J.F., and I. Lonnroth.** 1988. Purification and characterization of two forms of toxin B produced by Clostridium difficile. FEBS Lett. 233:417-420.

184. **Tsuzuki, K., N. Yokosawa, B. Syuto, I. Ohishi, N. Fujii, K. Kimura, and K. Oguma.** 1988. Establishment of a monoclonal antiboby recognizing an antigenic site common to Clostridium botulinum type B, C1, D, and E toxins and tetanus toxin. Infect. Immun. 56:898-902.

185. **van Heyningen, W.E.** 1974. Gangliosides as membrane recptors for tetanus toxin, cholera toxin and serotonin. Nature (London) 249:415-417.

186. **Vawter, L.R.** 1942. Recovery of Clostridium sordellii from cattle and sheep. Amer. J. Vet. Res. 3:382-386.

187. **Weinstein, L., and M.A. Barza.** 1973. Gas gangrene. New. Engl. J. Med. 289:1129-1131.

188. **White, A.B., and J. Mellanby.** 1969. The separation of neuraminidase activity from other pathological activities of a culture filtrate of Clostridium sordellii CN 3903. J. Gen. Microbiol. 56:137-141.

189. **Wilkins, T., M. Ehrich, R. Van Tassell, and E. Balish.** 1980. Studies on the clostridial toxin associated with antibiotic-induced pseudomembranous colitis, p.411-417. In D. Eaker and T. Wadstrom (ed.), Natural Toxins. Pergamon

Press, New York.

190. **Wilkins, T., H. Krivan, B. Stiles, R. Carman, and D. Lyerly.** 1985. Clostridial toxins active locally in the gastrointestinal tract, p.230-241. In D. Evered and J. Whelan (ed.). Microbial toxins and diarrhoeal disease, Ciba Symposium 112, Pitman, London.

191. **Willey, S.H., and J.G. Bartlett.** 1979. Cultures for Clostridium difficile in stools containing a cytotoxin neutralized by Clostridium sordellii antitoxin. J. Clin. Microbiol. 10:880-884.

192. **Willis, T.A.** 1969. Clostridia of Wound Infections. Butterworth, London.

193. **Yamakawa, K., S. Nakamura, and S. Nishida.** 1985. Separation of two cytotoxins of Clostridium sordellii strains. Microbiol. Immunol. 29:553-557.

194. **Yamakawa, K., N. Tanabe, Y. Okada, S. Nishida, and S. Nakamura.** 1983. Rabbit ileal loop responses to Clostridium sordellii strains. Microbiol. Immunol. 27:807-809.

195. **Yamamoto, T., T. Nakazawa, T. Miyata, A. Kaji, and T. Yokota.** 1984. Evolution and structure of two ADP-ribosylation enterotoxins, Escherichia coli heat-labile toxin and cholera toxin. FEBS Lett. 169:241-246.

Section III. Purification and Characterization of *Clostridium sordellii* Hemorrhagic Toxin and Cross-Reactivity with *Clostridium difficile* Toxin A (Enterotoxin).

ABSTRACT

Hemorrhagic toxin (HT) was purified from *Clostridium sordellii* culture filtrate. The purification steps included ultrafiltration through an XM-100 membrane filter and immunoaffinity chromatography using a monoclonal antibody to toxin A of *Clostridium difficile* as the ligand. Toxin HT migrated as a major band with molecular weight of 525,000 and a minor band at 450,000 on non-denaturing gradient PAGE. By SDS-PAGE the molecular weight was estimated at 300,000. Isoelectric focusing indicated an apparent pI of 6.1. HT was cytotoxic for cultured cells, lethal for mice by intraperitoneal injection and elicited an accumulation of hemorrhagic fluid in rabbit ileal loops. Immunodiffusion analysis revealed a reaction of partial identity between toxins A and HT. Immunological cross-reactivity between these toxins was further demonstrated by immunoblotting and by neutralization of HT biological activity with antibodies to toxin A. A sensitive indirect ELISA was used to examine the affinity involved in homologous and heterologous antigen-antibody interactions. Our findings show that toxin HT has biological activities and immunological properties similar to toxin A; however the toxins are not identical.

INTRODUCTION

Clostridium sordellii is one of six clostridia associated with clostridial myonecrosis (gas gangrene) in man (15), as well as in domestic animals (9). More recently it has been recognized as a causal agent of diarrhea and enterotoxemia in cattle and sheep (2-4, 30). C. sordellii also was once suspected to be the causative agent of pseudomembranous colitis (PMC) in humans. This was because the cytotoxicity of stool filtrates from PMC patients was neutralized by C. sordellii antitoxin (1, 18, 36). However, researchers were unable to isolate C. sordellii from stool samples of patients with PMC. Clostridium difficile isolated from these patients was later shown to be the cause of PMC in humans (8, 13). The cross-neutralization of C. difficile toxins by C. sordellii antitoxin was a fortuitous cross-reaction that led to this discovery. Two toxins, A and B, have been purified from culture supernatants of toxigenic strains of C. difficile (7, 40, 41). Both toxins are cytotoxic to tissue cultured mammalian cells, although toxin B is at least 1000-fold more cytotoxic. Toxin A is an enterotoxin, causing fluid accumulation with hemorrhage in the small intestine and cecum of animals (19, 24, 27). Both of the toxins produced by C. difficile are neutralized by C. sordellii antitoxin. This suggests that C. sordellii produces toxins immunologically related to the toxins

produced by C. difficile (1, 11, 35, 36).

The production of two toxins with distinctive biological activity by C. sordellii was first described by Arseculeratne et al. (5), who noted that intradermal injections of cell free extracts into guinea-pigs caused edema and hemorrhage. The edematous and hemorrhagic activities were shown to be independent, with the toxin which caused the edema being more lethal (Lethal Toxin, designated LT) than the hemorrhagic toxin (designated HT). It has been reported that culture supernatants are toxic to cultured cells and induce a hemorrhagic response in rabbit ileal loops (28, 29, 43, 44). The hemorrhagic toxin (HT) can be separated from the highly cytotoxic toxin (LT) by ion exchange chromatography (43). However the relatedness of these two toxins to the toxins of C. difficile was not studied by these authors. Popoff recently purified toxin LT and showed that it is similar to toxin B of C. difficile (31).

We report on the purification of the C. sordellii hemorrhagic toxin (toxin HT). Our findings show that this toxin has physicochemical characteristics and biological activities similar to those of C. difficile toxin A (enterotoxin).

MATERIALS AND METHODS

Protein determination. Protein concentrations were estimated by the method of Bradford (10) with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine gamma globulin was the standard.

Bacterial strains. C. sordellii VPI strains 9048 (Tox^+) and 7319 (Tox^-) were obtained from the anaerobe collection of the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University (Blacksburg) and were identified by L.V. Moore, E.P. Cato and W.E.C. Moore by methods in the V.P.I. Anaerobe Laboratory Manual (14). Cells were grown in 2-liter brain heart infusion dialysis flasks for 96 h at 37°C as described for the production of C. difficile toxins (40). This method was originally described by Sterne and Wentzel for the production of C. botulinum toxin (38).

C. difficile antiserum and toxin A antibodies. Rabbit and goat antisera against crude C. difficile VPI 10463 culture filtrate were produced as previously described (12). Affinity-purified goat antibody and PCG-4 mouse monoclonal antibody (PCG-4 MAb) were also prepared as previously described (24).

Purification of toxin HT by immunoaffinity chromatography. Monoclonal antibody to toxin A (PCG-4 MAb) was partially purified from ascites by 45% $(NH_4)_2SO_4$ precipitation and was coupled to Affi-Gel 10 (Bio-Rad) as recommended by the manufacturer. About 15 mg of protein were bound per ml of gel. Culture filtrate from C. sordellii VPI 9048 (500 ml) was concentrated to 100 mls by ultrafiltration on a XM-300 membrane (Amicon Corp., Lexington, Mass.) and washed with two volumes of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl (TBS). The sample was passed through a column (2.5 by 20 cm) containing 5 ml of PCG-4 MAb-gel, and the gel was washed with 100 bed volumes of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.50 M NaCl to remove the unbound material. The bound toxin was eluted with 1.5 bed volumes of 5 M $MgCl_2.6H_2O$ (in TBS), dialyzed against TBS and concentrated by ultrafiltration to 0.5 ml with a Centriprep-30 concentrator (Amicon Corp.). The concentrated toxin preparation was stored at 4^oC.

Crossed immunoelectrophoresis and immunodiffusion analysis. Crossed immunoelectrophoresis was performed as described before (40). Ouchterlony double-immunodiffusion analysis was done as described previously (16).

Analytical isoelectric focusing. Analytical isoelectric

focusing was performed in thin-layer isoelectric focusing polyacrylamide gels with the LKB 2117 Multiphor apparatus and commercial polyacrylamide gel plates (pH 3.5 to 9.5) as recommended by the manufacturer (Pharmacia, Inc., Piscataway, NJ). Isoelectric point standards were purchased from Pharmacia, Inc.

Polyacrylamide gel electrophoresis (PAGE). Nondenaturing PAGE was done in two types of gels. Gradient PAGE was done as previously described (16). Molecular weight was estimated with a high-MW calibration kit (Pharmacia). PAGE was also done in a discontinuous gel system according to procedures described before (23). SDS-PAGE was done according to the general procedures of Laemmli (17) as described before (23).

Temperature stability. Immunoaffinity purified toxin HT (0.15 mg/ml) and culture filtrate (1.30 mg/ml) were stored at -20, 4, 20 to 25 and 37^{o}C. After 72 h samples were examined for loss of cytotoxicity and lethality. The purified toxin and the crude culture filtrate were also incubated at 56^{o}C , and samples were taken at time intervals (0, 5, 10, 15, 30, and 60 min) and examined for loss of cytotoxic and lethal activities.

pH stability. Purified toxin HT (0.75 mg/ml) was diluted

1:10 in the following pH buffer systems and incubated for two hours at room temperature: 0.2 M glycine-HCl (pH 2.0), 0.05 M acetate (pH 4.0), 0.05 M Tris-hydrochloride (pH 7.5), and 0.2 M glycine-NaOH (pH 10.0). The samples then were diluted 1:1 in 0.2 M Tris-hydrochloride buffer (pH 7.5), and examined for loss of cytotoxic and lethal activities.

Immunoblotting. Samples from polyacrylamide gels were blotted onto nitrocellulose membranes by procedures described previously (23).

Production of C. sordellii antiserum. Toxoid was prepared by incubating crude culture filtrate from C. sordellii VPI strain 9048 in formaldehyde (0.1% final concentration) for three hours at 37°C. New Zealand White female rabbits (Hazleton Research Animals, Denver, PA.) were injected subcutaneously each week with 2.5 ml of formalinized culture filtrate mixed with an equal volume of Freunds incomplete adjuvant (Sigma Chemical Co.). Antitoxin was detected after the sixth injection and reached maximum titers, as determined by crossed immunoelectrophoresis with crude culture filtrate in the first dimension, 24 weeks after the start of injections. At that time the rabbit was bled-out by cardiac puncture.

ELISA. An indirect ELISA for the detection of toxin HT was done as previously described for the detection of toxin A of C. difficile (27). Affinity-purified goat antibodies or mouse ascites containing the MAb PCG-4 to toxin A were used as the detecting antibody. The amount of toxin HT present in the culture filtrate was determined by comparison to a standard curve with known concentrations of homogeneous toxin HT.

Biological assays. (i) Cytotoxicity assay. The cytotoxicity assay was determined as described previously (6). The tissue culture dose (TCD_{100}) was defined as the reciprocal of the highest dilution which rounded 100% of the CHO-K1 cells. Neutralization of toxin HT with C. difficile toxin A antibodies was performed as previously described (20). The neutralization titer was expressed as the reciprocal of the dilution which completely inhibited the rounding of all cells.

(ii) Lethality assay. Lethality in mice was determined as previously described (40). The LD_{100} was defined as the highest dilution of toxin which killed 100% of the mice within 36 h. Neutralization was determined by mixing two fold dilutions of the antitoxins with two fold LD_{100} doses of the toxin. After incubation at room temperature for one hour, 0.5 ml of each mixture was administered to mice by

intraperitoneal injection. The titer of each antitoxin was defined as the reciprocal of the highest dilution of antitoxin at which all mice survived the LD_{100}.

(iii) Ligated intestinal loops. Ligated intestinal loops were prepared as described by Lyerly et al. (22). C. difficile toxin A (10ug) served as positive control. Ratios greater than 1 were considered positive. For the neutralization assay, 100 ug of toxin HT or 0.5 ml of C. sordellii 9048 culture filtrate (1:2.5 dilution in phosphate-buffered saline were mixed with an equal volume of undiluted PCG-4 MAb (ascites) and incubated for 30 min at room temperature. After incubation 1 ml samples of each mixture were injected into ileal loops. The enterotoxic response was determined as described above.

RESULTS

Purification of toxin HT. Toxin HT was specifically and quantitatively removed from the concentrated culture filtrate by the monoclonal antibody to toxin A of C. difficile coupled to Affi-Gel 10 (Fig. 1). The toxin was eluted with 5M $MgCl_2.6H_2O$ and showed a single immunoprecipitin arc by crossed immunoelectrophoresis against antiserum to C. sordellii culture filtrate (Fig. 1c). Table 1 shows the percent recovery of toxin HT as determined by indirect ELISA.

We also attempted to remove the toxin HT from the crude culture filtrate using bovine thyroglobulin immobilized on Affi-Gel 15 as described for the purification of C. difficile toxin A (16). Toxin HT did bind to the receptor column and could be eluted at 37°C; however, the yield was very low and the toxin was contaminated with degraded thyroglobulin (data not shown).

Characterization of toxin HT. The preparation of toxin HT which eluted from the PCG-4 MAb column showed one major band and one minor band on gradient PAGE. The major band had an estimated native molecular weight of 525,000 daltons whereas the molecular weight of the minor band was 450,000 daltons (Fig. 2). The toxin HT preparation migrated on SDS-PAGE as one major band with an estimated molecular weight of 300,000 and several faster migrating bands (Fig. 3). By isoelectric focusing the purified toxin preparation focused as a single band at pH 6.10 (Fig. 4).

To identify toxin HT in the crude culture filtrate, immunoblots were made with culture filtrate from a toxigenic C. sordellii strain (VPI 9048). Immunoblots were also done with culture filtrate of a non-toxigenic strain (VPI 7319) and with affinity purified toxin HT. Under non-denaturing conditions the PCG-4 monoclonal antibody reacted with both bands in the toxin HT preparation and in the culture

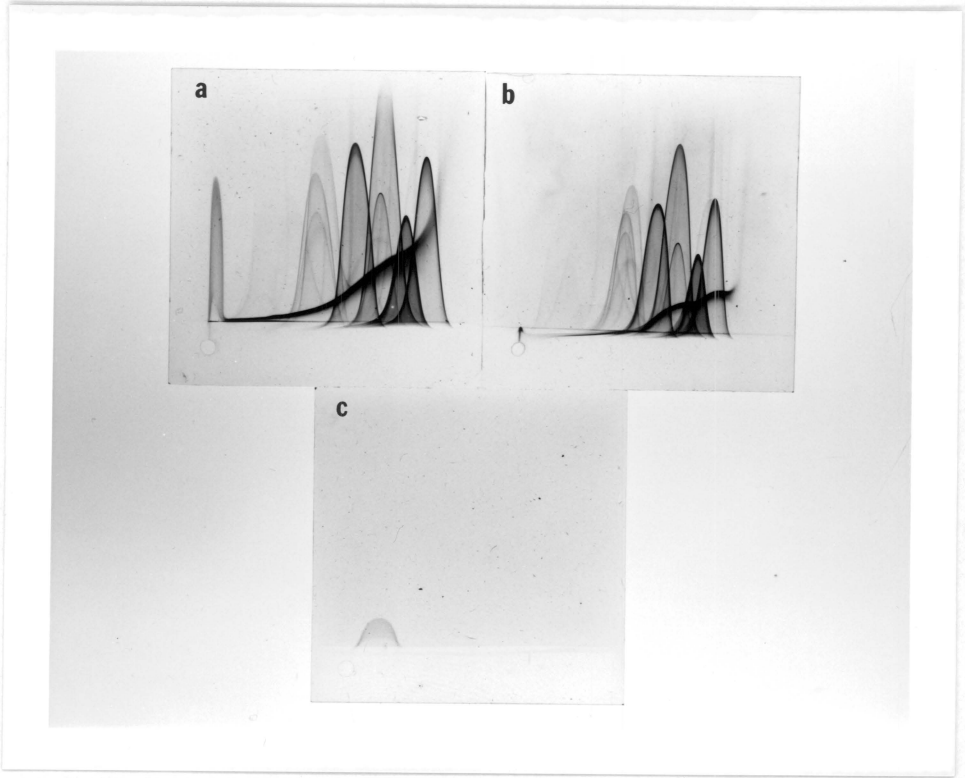

Figure. 1. Analysis by crossed immunoelectrophoresis of toxin HT purified by immunoaffinity chromatography on PCG-4 MAb-Affi-Gel 10. Culture filtrate from C. sordellii VPI 9048 was adsorbed with the gel, and the bound toxin was eluted from the gel. (a) Culture filtrate before immunoadsorption (80 ug). (b) Culture filtrate after immunoadsorption (75 ug). The arrow indicates the disappearance of the toxin HT immunoprecipitin arc, demonstrating the removal of the toxin from culture filtrate. (c) Toxin HT (1.5 ug) eluted from the gel with 5M $MgCl_2.6H_2O$ in 0.05M Tris hydrochloride-0.15M NaCl, pH 7.5. The upper portion of each plate contained 250 ul of rabbit antiseum against culture filtrate from C. sordellii VPI 9048.

Table 1. Purification of *C. sordellii* toxin HT.

	Vol (ml)	Protein		% Recovery[a]
		mg/ml	Total mg	
Culture filtrate	500	1.30	650	100
Ultrafiltration	100	2.50	250	33
Concentrated affinity column eluate	0.5	0.70	0.35	8.5

[a]Percent of toxin HT recovered from starting material found in culture filtrate as determined by ELISA.

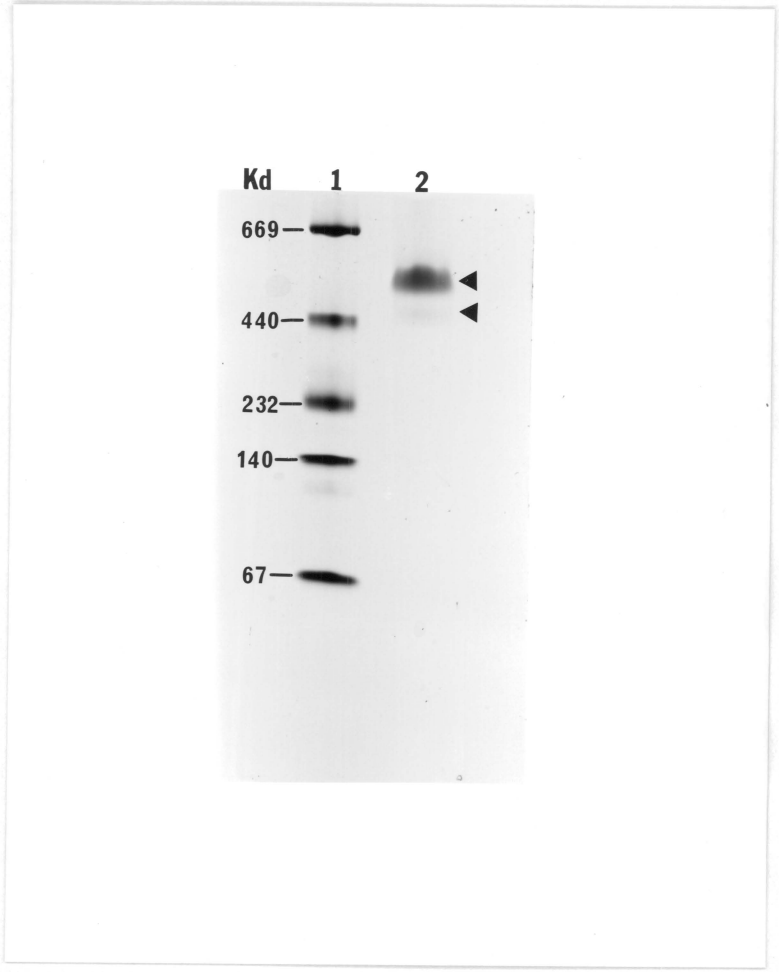

Figure 2. Gradient (4 to 30%) PAGE of purified toxin HT. Lane 1, M.W. standards (Pharmacia); lane 2, purified toxin HT (25 ug). Arrows in lane 2 point to the major and minor bands.

filtrate of the toxigenic strain (Fig. 3, lanes 1 and 2). The monoclonal antibody did not react with any antigens in the culture filtrate from the non-toxigenic strain. Affinity purified antibodies to toxin A also reacted strongly with both bands in the toxin HT preparation (data not shown). The denatured toxin observed by SDS-PAGE was analyzed by immunoblotting to determine whether the minor bands were reactive with the monoclonal (PCG-4) and affinity purified antibodies to toxin A. Each of the antibodies to toxin A reacted with the major band and also with the minor bands (Fig. 3).

Immunodiffusion analysis was performed to examine the relationship between toxin HT and toxin A of C. difficile. Affinity purified antibodies to toxin A reacted with toxin HT and formed a precipitin line that showed partial identity with the C. difficile toxin (Fig. 5).

An indirect ELISA procedure was used to study the reaction of toxin A antibodies with toxin HT. The response observed with the MAb PCG-4 as the detecting antibody is shown in figure 6. When equal concentrations of toxin HT and toxin A were tested in the ELISA the titers of toxin HT were lower compared to those obtained with toxin A. The results observed with the affinity-purified goat antibody to toxin A were similar to those seen with the MAb PCG-4 (data not shown).

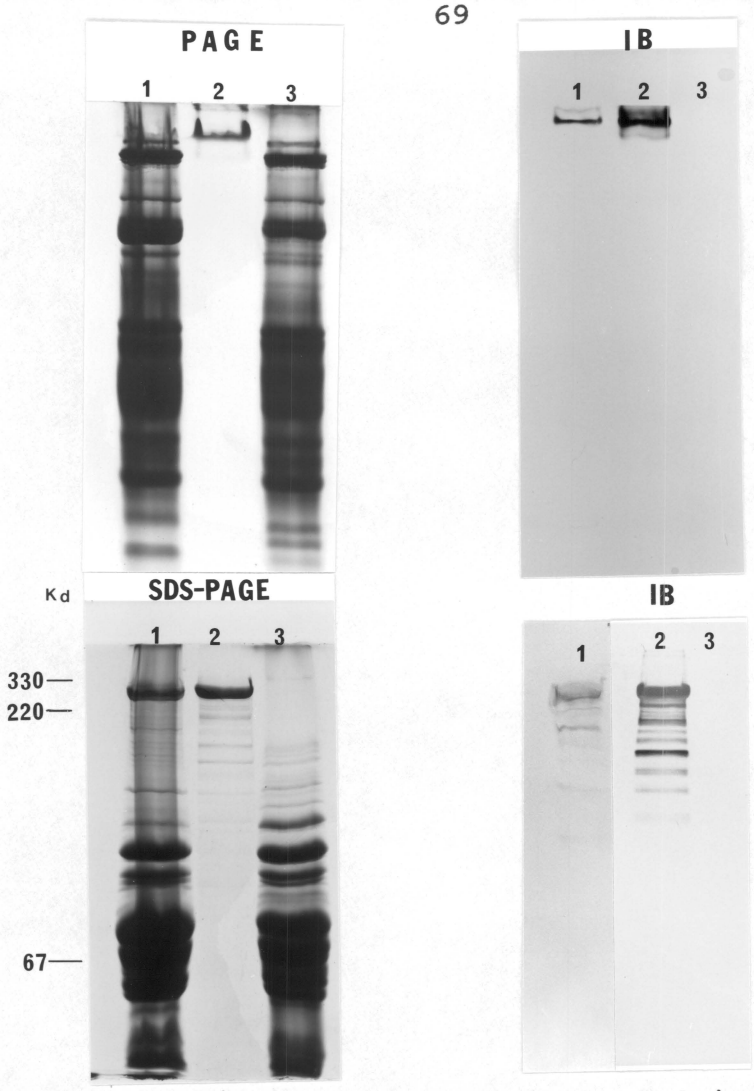

Figure 3. Analysis by PAGE, SDS-PAGE, and immunoblots of (1)toxigenic C. sordellii VPI 9048 culture filtrate (250 ug); (2) purifiedtoxin HT (8 ug); (3) non-toxigenic C. sordellii VPI 7319 culturefiltrate (250 ug). PAGE and SDS-PAGE were performed in 4% stacking-7.5% running gels. After electrophoresis the proteins were either stained with Coomassie brillant blue R-250 or blotted onto nitrocellulose for immunoblot analysis with monoclonal antibody specific to toxin A (MAb PCG-4). Molecular weight markers appear to the left of lower panels.

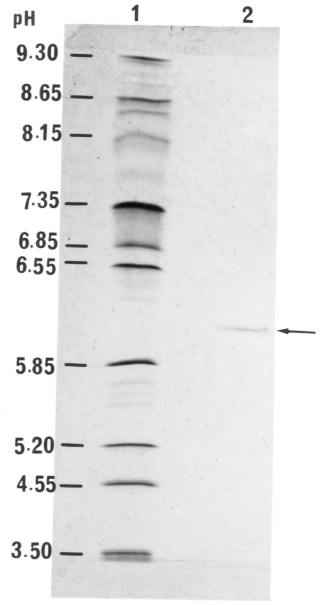

Figure 4. Estimation of toxin HT pI by analytical thin-layer isoelectric focusing in polyacrylamide gel (LKB). Lane 1, pI markers (Pharmacia); lane 2, toxin HT (2 ug).

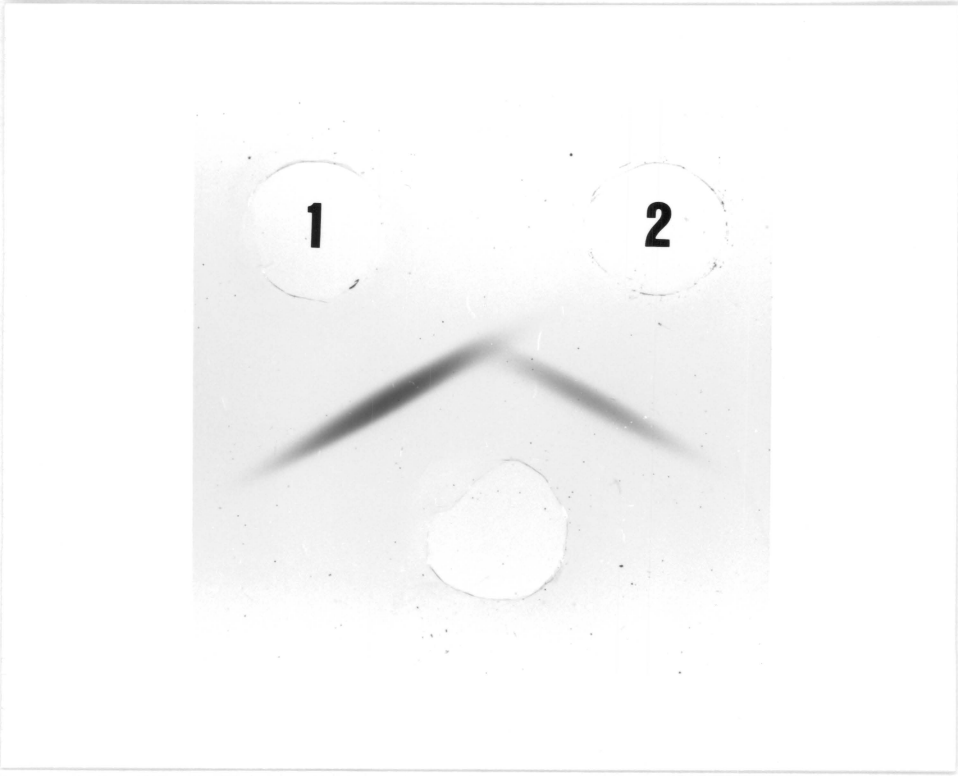

Figure 5. Immunodiffusion analysis of toxin A and toxin HT. Center well contained 20 ul of affinity-purified antibodies to toxin A. Well 1 contained 20 ug of toxin A; well 2 contained 20 ug of affinity purified toxin HT.

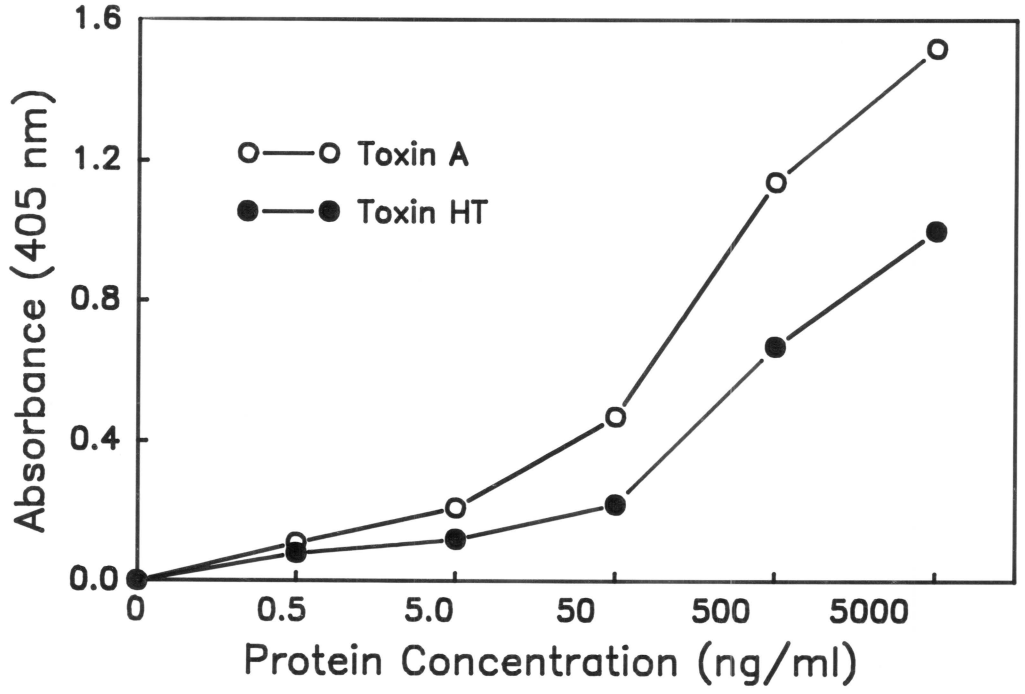

Figure 6. Comparative quantitation of toxin A and toxin HT in the indirect ELISA using antibodies to C. difficile toxin A. The detecting antibody consisted of a 1/1000 dilution of a monoclonal antibody specific to toxin A (MAb PCG-4).

Stability of toxin HT at different temperatures and pH. Toxin preparations were stable from -20 to 37°C. Purified toxin HT and culture filtrate were completely inactivated after incubation for 5 min at 56°C. Toxin HT was stable at pH 4.0 to 10.0, and completely inactivated at pH 2.0.

Biological Activity. The biological activities of toxin HT are listed in Table 2. Purified HT was cytotoxic and caused the same type of cell rounding response in CHO-K1 cells as toxin A of C. difficile. The specific activity of purified toxin HT in the tissue culture assay was 2.0 x 10^3/mg, whereas the specific activity of the culture filtrate was 1.0 x 10^3/mg. The specific activity in the mouse lethality assay was 8.3 x 10^3/mg and the specific of the culture filtrate was 3.2 x 10^4/mg (due to the presence of toxin LT).

Injection of purified toxin HT in rabbit intestinal loops elicited an accumulation of a viscous hemorrhagic fluid (Table 3). Culture filtrate from the toxigenic C. sordellii strain also elicited a similar response in the intestinal loop assay. No accumulation of fluid or hemorrhage was observed in the intestinal loops injected with culture filtrate from the non-toxigenic strain.

Affinity purified antibody to toxin A neutralized the cytotoxicity and lethality for mice (antibody titers of 128

and 1024 respectively) of toxin HT. These results indicate that toxin LT was completely removed from the HT preparation since affinity purified antibody to toxin A does not neutralize the cytotoxic and lethal activities of toxin LT. Affinity purified antibody to toxin A also neutralized the enterotoxic activity of HT. The PCG-4 MAb did not neutralize the cytotoxicity or lethality of HT but did neutralize the enterotoxicity (Table 3).

Table 2. Comparison of toxin HT and toxin A.

Property	Toxin HT	Toxin A[a]
Native molecular weight[b]	525,000	550,000
Denatured molecular weight	300,000	300,000
Isoelectric point	6.10	5.2 - 5.7
Temperature stability	Stable at -20 to 37°C, inactivated at 56°C.	
pH stability	Stable at pH 4.0 to 10.0	
TCD_{100}	500 ng	10 ng
LD_{100}	120 ng	90 ng
Enterotoxic[c] dose	100 ug (Actual) 2 ug (Corrected)	1 ug

[a]Toxin A purified by sequential ammonium sulfate precipitation, ion exchange chromatography, and precipitation at pH 5.6 (21, 23, 40).

[b]A minor band with an estimated molecular weight of 450,000 daltons was also observed in the toxin HT preparation.

[c]Minimum dose which induced a positive response in rabbit ileal loops.The corrected dose is based on the amount of toxin HT in the culture filtrate as determined by ELISA.

Table 3. Response in rabbit intestinal loops.

Sample	Vol/length ratio[a]
C. sordellii VPI 9048 culture filtrate (1/10 dilution)	1.48 ± 0.40
C. sordellii VPI 9048 culture filtrate + PCG-4 MAb	0.02 ± 0.01[b]
C. sordellii VPI 7319 culture filtrate	0.02 ± 0.01
Toxin HT (100 ug)	1.06 ± 0.18
Toxin HT (100 ug) + PCG-4 MAb	0.02 ± 0.01[c]
Toxin A (10 ug)	1.81 ± 0.22

[a]The volume/length ratios were determined 16 h after injection and are expressed as the mean ±1 standard error for four test loops injected per sample.

[b,c]Culture filtrate (1/2.5 dilution) or toxin HT was mixed with undiluted PCG-4 ascites. Ascites against another clostridial species did not have any effect on the enterotoxic activity of the culture filtrate from the toxigenic strain of C. sordellii or toxin HT.

DISCUSSION

This study represents the first description of a purification scheme for toxin HT from C. sordellii and the first direct comparison of this toxin to toxin A of C. difficile. We purified toxin HT from culture filtrate of C. sordellii using a single immunoaffinity step involving a monoclonal antibody against toxin A. This antibody binds with less avidity to toxin HT than to toxin A, which accounts for the differences in elution of the toxins from the PCG-4 MAb column. HT readily eluted from the MAb-gel with $MgCl_2$ whereas toxin A requires harsher conditions such as pH extremes (23). The purified toxin HT was homogeneous by crossed-immunoelectrophoresis and analytical isoelectric focusing. This preparation had a major and a minor band when analyzed by analytical PAGE under non-denaturing conditions. This minor band reacted with toxin A antibodies and was detected in the culture filtrate. Thus, it is not a contaminant and does not result from the purification procedure.

Toxin HT and toxin A exhibit a number of similarities. Both toxins have estimated molecular weights in excess of 300,000 as determined by SDS-PAGE. The very large size of toxin HT and toxin A is unusual and this has led many investigators to suggest that the toxins have subunit structures. The high molecular weight of toxin A is

supported, however, by the finding that its gene has an open reading frame larger than 7.1 kilobases (J. L. Johnson, personal communication) which means that the resulting polypeptide is larger than 290,000 daltons.

Several faster migrating bands are observed when toxin HT and toxin A are subjected to denaturing conditions and analyzed by SDS-PAGE (23). These minor bands react with both monoclonal and affinity-purified antibodies to toxin A in immunoblots. The appearance of these bands is not inhibited by protease inhibitors (R.D. Martinez and T.D. Wilkins, unpublished results), but it is still unclear whether they are the product of proteolysis. The fact that this occurs with both toxin HT and toxin A indicates that this is a common characteristic of these toxins.

Most strains of C. sordellii produce low amounts of toxins and this makes purification by classical techniques extremely difficult. C. sordellii 3703 has been reported to produce large amounts of these toxins (28, 29, 43, 44), but this strain was unavailable to us for comparison.

We attempted to purify toxin HT by use of the receptor-affinity column procedure which has been used recently for the purification of toxin A (16). In this procedure toxin A binds to the trisaccharide Galα1-3Galβ1-4GlcNAc on bovine thyroglobulin at 4^{o}C and then is eluted by increasing the temperature to 37^{o}C. When toxin HT was purified in this

manner the resulting preparation was contaminated with thyroglobulin. C. sordellii is considerably more proteolytic than C. difficile (14) and we suspect that the thyroglobulin present in the HT preparation resulted from proteolytic cleavage of immobilized thyroglobulin. However, this work and the work by Price et al. (32) indicates that the two toxins may have similar receptors.

Affinity purified toxin HT retained its biological activities. These activities are similar to those observed with toxin A (25, 40, 41) and included cytotoxicity (cell rounding), lethality and enterotoxicity. The cytotoxic activity of HT is significantly less than the cytotoxicity of toxin A. This may be due to partial inactivation of the toxin during the elution from the antibody column with $MgCl_2$ since toxin A purified by immunoaffinity chromatography also loses a significant amount of cytotoxic activity (23). However, the lethal activity of the affinity purified toxin HT is comparable to that of toxin A. We could not follow the specific activity of the cytotoxicity or lethality through the purification scheme because the other C. sordellii toxin (LT) has these activities and masks the action of HT in such assays (31). Toxin HT elicited a positive response (with hemorrhage, mucus and fluid accumulation) in the rabbit ileal loop assay that is identical to the type of reaction caused by toxin A (22). The hemorrhagic fluid response

induced by toxins HT and A appears to be a common mode of action for both toxins and is not observed with cholera toxin and the heat-labile enterotoxin of E. coli which cause the accumulation of a watery fluid (34). The purified toxin HT appears to have lost more than 90% of its enterotoxic activity; we base this on the amount of toxin HT detected by ELISA in the culture filtrate and comparison of it enterotoxicity to the enterotoxicity of purified toxin HT. If this loss were taken into account, the enterotoxic specific activity of toxin HT would be similar to that of toxin A.

Antibodies to toxin A did not significantly neutralize the cytotoxic or lethal activities of the C. sordellii culture filtrate. This indicates that toxin LT is a more potent toxin accounting for most of the cytotoxic and lethal activities present in the culture filtrate. This situation is analogous to that seen with culture filtrates of C. difficile treated with toxin A antibodies (20). In that instance neutralization of toxin A does not neutralize the cytotoxic or lethal effects due to toxin B. On the other hand, the monoclonal antibody (PCG-4) to toxin A did completely neutralize the enterotoxicity of the C. sordellii culture filtrate; this indicates that toxin HT is responsible for all the enterotoxicity, just as toxin A is responsible for all of the enterotoxicity of the C.

difficile culture filtrates (26).

Previous investigators have documented the fact that immunological cross-reactivity exists between the toxins produced by *C. difficile* and by *C. sordellii* (1, 11, 33, 36). Antisera against either of the toxic culture filtrates neutralize the action of toxins present in the culture filtrate of the other organism, although usually in a less potent manner, suggesting that this is due to a lack of serological specificity (6, 12, 42). Analysis by immunodiffusion revealed a reaction of partial identity between toxin A and toxin HT. This supports the presence of common antigenic determinants between the two toxins. The antigenic differences between the toxins also were evident from the ELISA results showing that the toxin A antibodies did not react as strongly with HT as toxin A, indicating a lower binding affinity of the toxin A antibodies to cross-reactive epitopes present on toxin HT.

The production of similar toxins by different species and even different genera of bacteria is not unusual. The oxygen-labile hemolysins, for example, are closely related proteins that are produced by members of the following genera: *Bacillus*, *Clostridium*, *Listeria* and *Streptococcus* (37). This type of phenomenon is also seen with clostridial species; *C. perfringens* and *C. spiroforme* produce binary toxins that share many immunochemical and physicochemical

features (39). Comparison of closely related toxins produced by different organisms can yield important information since the portions of the molecules which are most conserved are normally the active and binding sites. Our future aim is to compare the nucleotide sequence of the genes coding for the toxins of C. sordellii to those of C. difficile so that the conserved areas can be identified and studied in detail. We are also interested in any differences in biological activity that may have occurred due to genetic changes.

In conclusion, our work and the work of Popoff (31) show that toxins HT and LT of C. sordellii correspond to toxins A and B of C. difficile. Although the toxins are not identical, they have retained remarkable immunological similarities as well as physicochemical and biological properties.

LITERATURE CITED

1. **Allo, M., J. Silva. R. Fekety, G.D. Rifkin, and H. Waskin.** 1979. Prevention of clindamycin-induced colitis in hamsters by Clostridium sordellii antitoxin. Gastroenterology 76:351-355.

2. **Al Mashat, R.R., and D.J. Taylor.** 1983. Production of diarrhoea and enteric lesions in calves by the oral inoculation of pure cultures of Clostridium sordellii. Vet. Rec. 112:141-146.

3. **Al Mashat, R.R., and D.J. Taylor.** 1983. Clostridium sordellii in enteritis in an adult sheep. Vet. Rec. 112:19.

4. **Al Mashat, R.R., and D.J. Taylor.** 1983. Bacteria in enteric lession in cattle. Vet. Rec. 112:5-10.

5. **Arseculeratne, S.N., R.G. Pannabokke, and S. Wijesundera.** 1969. The toxins responsible for the lesions of Clostridium sordellii gas gangrene. J. Med. Microbiol. 2:37-53.

6. **Aswell, J.E., M. Ehrich, R.L. Van Tassell, C.-C. Tsai, L.V. Holdeman, and T.D. Wilkins.** 1979. Characterization and comparison of Clostridium difficile and other clostridial toxins, p.272-275. In D. Schlessinger (ed.), Microbiology-1979. American Society for Microbiology, Washington, D.C.

7. **Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of Clostridium difficile causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. 2:629-635.

8. **Barlett, J.G., T.-W. Chang, M. Gurwith, S.L. Gorbach, and A.B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin producing clostridia. N. Engl. J. Med. 298:531-534.

9. **Blood, D.C., J.A. Henderson, and O.M. Radostits.** 1979. Veterinary medicine, p.445-447. Balliere Tindal, London.

10. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

11. **Chang, T.-W., S.L. Gorbach, and J.G. Bartlett.** 1978. Neutralization of Clostridium difficile toxin by Clostridium

sordellii antitoxins. Infect. Immun. 52:418-422.

12. **Ehrich, M., R.L. Van Tassell, J.M. Libby, and T.D. Wilkins.** 1980. Production of Clostridium difficile antitoxin. Infect. Immun. 28:1041-1043.

13. **George, R.H., J.M. Symonds, F. Dimock, J.D. Brown, Y. Arabi, N. Shinagawa, M.R.B. Keighley, J. Alexander-Williams, and D.W. Burdon.** 1978. Identification of Clostridium difficile as a cause of pseudomembranous colitis. Br. Med. J. 1:695.

14. **Holdeman, L.V., E.P. Cato, and W.E.C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.

15. **Joklik, W.K., H.P. Willet, and D.B. Amos (ed.).** 1984. Zinsser microbiology. p.698-707. Appleton-Century-Crofts, Norwalk, Conn.

16. **Krivan, H.C., and T.D. Wilkins.** 1987. Purification of Clostridium difficile toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. 55:1873-1877.

17. **Laemmli, U.K.** 1970. Cleavage of Structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) 227:680-685.

18. **Larson, H.E., and A.B. Price.** 1977. Pseudomembranous colitis: presence of clostridial toxin. Lancet ii:1312-1314.

19. **Libby, J.M., B.S. Jortner, and T.D. Wilkins.** 1982. Effects of the two toxins of Clostridium difficile in antibiotic-associated cecitis in hamsters. Infect. Immun. 36:822-829.

20. **Libby, J.M., and T.D. Wilkins.** 1982. Production of antitoxins to two toxins of Clostridium difficile and immunological comparison of the toxins by cross-neutralization studies. Infect. Immun. 35:374-376.

21. **Lyerly, D.M., H.C. Krivan, and T.D. Wilkins.** 1988. Clostridium difficile: its disease and toxins. Clin. Microbiol. Rev. 1:1-18.

22. **Lyerly, D.M., D.E. Lockwood, S.H. Richardson, and T.D. Wilkins.** 1982. Biological activities of toxins A and B of Clostridium difficile. Infect. Immun. 35:1147-1150.

23. **Lyerly, D.M., C.J. Phelps, J. Toth, and T.D. Wilkins.** 1986. Characterization of toxins A and B of Clostridium difficile with monoclonal antibodies. Infect. Immun. 54:70-76.

24. **Lyerly, D.M., C.J. Phelps, and T.D. Wilkins.** 1984. Monoclonal and specific polyclonal antibodies for immunoassay of Clostridium difficile toxin A. J. Clin. Microbiol. 21:12-14.

25. **Lyerly, D.M., M.D. Roberts, C.J. Phelps, and T.D. Wilkins.** 1986. Purification and properties of toxins A and B of Clostridium difficile. FEMS Microbiol. Lett. 33:31-35.

26. **Lyerly, D.M., K.E. Saum, D.K. MacDonald, and T.D. Wilkins.** 1985. Effects of Clostridium difficile toxins given intragastrically to animals. Infect. Immun. 47:349-352.

27. **Lyerly, D.M., N.M. Sullivan. and T.D. Wilkins.** 1983. Enzyme-lynked immunosorbent assay for Clostridium difficile toxin A. J. Clin. Microbiol. 17:72-78.

28. **Nakamura, S., H. Ogura, J. Tanaka, N. Tanabe, K. Yamakawa, M. Hatano, and S. Nishida.** 1984. Difference in susceptibility of various cell cultures to cytotoxic culture filtrates of Clostridium sordellii. Microbiol. Immunol. 28:493-497.

29. **Nakamura, S., N. Tanabe, K. Yamakawa, and S. Nishida.** 1983. Cytotoxin production by Clostridium sordellii strains. Microbiol. Immunol. 27:495-502.

30. **Popoff, M.R.** 1984. Bacteriological examination in enterotoxaemia of sheep and lamb. Vet. Rec. 114:324.

31. **Popoff, M.R.** 1987. Purification and characterization of Clostridium sordellii lethal toxin and cross-reactivity with Clostridium difficile cytotoxin. Infect. Immun. 55:35-43.

32. **Price, S.B., C.J. Phelps, T.D. Wilkins, and J.L. Johnson.** 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of Clostridium difficile. Curr. Microbiol. 16:55-60.

33. **Regh, J.E.** 1980. Cecal toxin(s) from guinea pigs with clindamycin-associated colitis, neutralization by Clostridium sordellii antitoxin. Infect. Immun. 27:387-390.

34. **Richards, K.L., and S.D. Douglas.** 1978.

Pathophysiological effects of Vibrio cholerae and enterotoxigenic Escherichia coli and their exotoxins on eucaryotic cells. Microbiol. Rev. 42:592-613.

35. **Rifkin, G.D., F.R. Fekety, and J. Silva.** 1978. Neutralization by Clostridium sordellii antitoxin of toxins implicated in clindamycin-induced cecitis in hamsters. Gastroenterology. 75:422-424.

36. **Rifkin, G.D., F.R. Fekety, J. Silva, and R.B. Sack.** 1977. Antibiotic-induced colitis. Implication of a toxin neutralised by Clostridium sordellii antitoxin. Lancet ii:1103-1106.

37. **Stephen, J., and R.A Pietrowski.** 1986. Bacterial toxins, 2nd ed., p.74-77. American Society for Microbiology, Washington, D.C.

38. **Sterne, M., and L.M. Wentzel.** 1950. A new method for the large scale production of high-titre botulinum formol-toxoid types C and D. J. Immunol. 35:1032-1040.

39. **Stiles, B.G., and T.D. Wilkins.** 1986. Clostridium perfringens iota toxin: synergism between two proteins. Toxicon 24:767-773.

40. **Sullivan, N.M., S. Pellett, and T.D. Wilkins.** 1982. Purification and characterization of toxins A and B of Clostridium difficile. Infect. Immun. 35:1032-1040.

41. **Taylor, N.S., G.M. Thorne, and J.G. Barlett.** 1981. Comparison of two toxins produced by Clostridium difficile. Infect. Immun. 34:1036-1043.

42. **Wilkins, T., M. Ehrich, R. Van Tassell, and E. Balish.** 1980. Studies on the clostridial toxin associated with antibiotic-associated pseudomembranous colitis, p.411-417. In D. Eaker and T. Wadstrom (ed.). Natural toxins. Pergamon Press, Inc., Elmsford, N.Y.

43. **Yamakawa, K., S. Nakamura and S. Nishida.** 1985. Separation of two cytotoxins of Clostridium sordellii strains. Microbiol. Immunol. 29:553-557.

44. **Yamakawa, K., N. Tanabe, Y. Okada, S. Nishida, and S. Nakamura.** 1983. Rabbit ileal loop responses to Clostridium sordellii strains. Microbiol. Immunol. 27:807-809.

Section IV. Comparison of *Clostridium sordellii* toxins HT and LT to toxins A and B of *Clostridium difficile*.

ABSTRACT

Clostridium sordellii produces two toxins, designated HT (Hemorrhagic Toxin) and LT (Lethal Toxin), that are similar to toxins A and B of *Clostridium difficile*. Examination of the physicochemical properties of toxin HT revealed remarkable similarities to toxin A. The specific biological activities of toxin HT were almost the same as toxin A in the cytotoxicity, lethality for mice and enterotoxicity assays, and their NH_2-terminal sequences shared close homology. Toxin LT exhibited properties similar to toxin B, and their NH_2-terminal sequences were very similar; however, toxin B was much more cytotoxic than toxin LT. Immunodiffusion analysis using specific antibodies revealed that although toxins B and LT shared major antigenic determinants, each also had unique epitopes. The results suggest that toxins B and LT have diverged more than toxins A and HT. In addition, the presence of common antigenic determinants between toxins HT and LT was demonstrated by immunoblotting using antibodies to the toxins of *C. difficile*.

INTRODUCTION

Several studies have implicated Clostridium sordellii as a cause of diarrhea and enterotoxemia in domestic animals (2, 29, 32), and more recently as an agent of toxic shock-like syndrome (11, J.A. McGregor et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1988). This bacterium was once suspected to cause pseudomembranous colitis (PMC) in humans because the cytotoxicity of stool filtrates from PMC patients was neutralized by C. sordellii antitoxin (1, 16, 34). However, researchers were unable to isolate C. sordellii from the stool samples of patients with PMC. This discrepancy was clarified when Clostridium difficile was isolated from fecal samples of patients with PMC (5, 10), and it was shown that the toxins produced by this organism are neutralized by C. sordellii antitoxin (1, 7, 33, 34). Two toxins, A and B, have been purified from culture supernatants of toxigenic strains of C. difficile (4, 39, 40). Both toxins are large proteins, which are lethal to animals and cytotoxic. Toxin A is a potent enterotoxin that elicits a hemorrhagic fluid response in the rabbit ileal loop assay (17, 20, 24).

C. sordellii produces two toxins that are similar to toxins A and B, which explains why C. sordellii antitoxin neutralizes the toxins of C. difficile. The toxins produced by C. sordellii have been designated HT (Hemorrhagic Toxin) and LT (Lethal Toxin) (3). We previously described the

purification of toxin HT by ultrafiltration and immunoaffinity chromatography using a monoclonal antibody to toxin A, and showed that toxin HT has biological activities and immunological properties similar to those of toxin A (26). Popoff has purified toxin LT and showed that it is immunologically related to toxin B (30). The toxins produced by C. difficile and by C. sordellii have retained similar physicochemical as well as immunological and biological properties; however, the toxins are not identical. Therefore, it was of interest to compare in more detail the properties of these toxins.

Our results demonstrate that the properties of toxins A and HT are remarkably similar, while toxins B and LT exhibit more differences. In addition, we show that C. sordellii toxins HT and LT share antigenic determinants, which suggests the possibility of common domains between these toxins.

MATERIALS AND METHODS

Protein Determination. Protein concentration was estimated by the method of Bradford (6) with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine gamma globulin (Bio-Rad) was the standard.

Bacterial strains. C. difficile VPI 10463 (Tox^+) and C.

sordellii VPI 9048 (Tox^+) were obtained from the culture collection of the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University (Blacksburg) and were identified by L.V. Moore, E.P. Cato, and W.E.C. Moore by methods described in the Virginia Polytechnic Institute Anaerobe Laboratory Manual (12). Cells were grown in 2-liter brain heart infusion dialysis flasks for 72 h at 37°C as described for the production of Clostridium botulinum toxin (37).

Antisera. Rabbit and goat antisera against crude C. difficile VPI 10463 culture filtrate was produced as previously described (9). Affinity purified goat antibody and monoclonal antibodies were also prepared as previously described (22). Rabbit antisera against crude C. sordellii VPI 9048 culture filtrate was produced as described previously (26).

Purification of toxin HT. Toxin HT was purified from the culture filtrate of C. sordellii VPI 9048 by ultrafiltration on a XM-300 membrane (Amicon Corp., Lexington, Mass.) and immunoaffinity chromatography using MAb PCG-4, a monoclonal antibody to toxin A, coupled to Affi-Gel 10 (Bio-Rad) as previously described (26). A partially purified preparation of toxin HT also was obtained, as described previously (41).

Partial purification of toxin LT. Partially purified toxin LT was obtained by a procedure similar to the one described by Yamakawa et al (41). C. sordellii VPI 9048 culture filtrate (100 ml) was concentrated to 10 ml by ultrafiltration on a XM-100 membrane (Amicon, Corp.). The concentrated culture filtrate was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 22 mM NaCl, and loaded onto 1 ml of DEAE-Sepharose CL-6B (Sigma) column (0.5 by 10 cm). The gel was washed with 25 bed volumes of 50 mM Tris-HCl, pH 7.5, containing 22 mM NaCl. Toxin LT was eluted with 3 bed volumes of 50 mM Tris-HCl, pH 7.5, containing 25 mM NaCl. To remove any contaminating HT the sample was passed through a column containing PCG-4 MAb affixed to Affi-Gel 10. The toxin preparation was stored at 4^{o}C.

PAGE. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was done in a discontinuous system as described before (21). Sodium dodecyl sulfate (SDS)-PAGE was done according to the general procedures of Laemmli (15) by methods previously described (21). To determine the proportion of toxin LT in the partially purified preparation, gels stained with Coomassie brilliant blue R-250 were scanned with a densitometer (model 620 video densitometer; Bio-Rad) in the reflectance mode.

Immunoblotting. Samples from polyacrylamide gels were blotted onto nitrocellulose membranes by procedures described previously (21).

ELISA. An indirect enzyme-linked immunosorbent assay (ELISA) for the detection of toxin HT was done as previously described for the detection of toxin A of C. difficile (25). MAb PCG-4 was used as the detecting antibody. The amount of toxin HT in the partially purified HT preparation was calculated from the linear portion of the ELISA standard curve obtained with known concentrations of toxin HT.

Crossed immunoelectrophoresis and immunodiffusion analysis. Crossed immunoelectrophoresis was performed as described before (39). Ouchterlony double-immunodiffusion analysis was done as described previously (26).

Amino acid analysis. Amino acid composition of purified toxin HT was performed in triplicate on 24-, 48-, and 72-h hydrolyzed samples by the method of Spackman et al. (35) as described before (21). The tryptophan content was determined spectrophotometrically by the Edelhoch technique (8).

Amino acid sequence. The amino-terminal sequence of toxins HT and LT were done according to the method of Matsudaira

(27), at the Protein Sequencing Center of the University of Virginia, Charlottesville, Va. Toxin preparations consisted of affinity-purified HT (30 ug) and partially purified LT (50 ug).

Biological assays. Cytotoxicity, lethality, rabbit intestinal loops and neutralization assays were performed by previously published methods (18, 20, 39).

RESULTS

Toxin purification and characterization. Toxin HT was specifically removed from the concentrated culture filtrate by the MAb to toxin A of C. difficile coupled to Affi-Gel 10 as we previously described (26). Toxin HT eluted from the PCG-4 MAb column was homogeneous as determined by crossed immunoelectrophoresis (Fig. 1). On nondenaturing PAGE the preparation of toxin HT showed one major band and a minor band (Fig 2). Toxin HT migrated on SDS-PAGE as one major band with an estimated molecular weight of 300,000 and several faster migrating bands (Fig. 3). The amino-terminal sequence of toxin HT was determined, and compared with the amino-terminal sequence of toxin A (C.H. Dove et al., submitted for publication). The amino-terminal sequence of toxins A and HT share close homology (Table 1).

The DEAE-Sepharose CL-6B column separated the toxins HT

and LT in C. sordellii culture filtrate. To remove any contaminating toxin HT the DEAE-Sepharose CL-6B fraction containing toxin LT was passed through a MAb PCG-4 Affi-Gel 10 column. Analysis by PAGE revealed at least four protein bands in the partially purified toxin LT preparation (Fig. 2). Several immunoprecipitin arcs were also observed by crossed-IEP (Fig. 1). A major band with an estimated molecular weight of 260,000 and several faster migrating bands were observed when the LT preparation was analyzed by SDS-PAGE (Fig. 3). Toxin LT represented 80% of the protein bands of the partially purified preparation, as determined by densitometric analysis. Immunoblots were made to identify toxin LT in the partially purified preparation. Antibodies to toxin B reacted only with the major protein band in the partially purified preparation (not shown). To further clarify its identity, the amino-terminal sequence of this 260,000-dalton protein band was determined and revealed close homology with the sequence recently published for toxin B (Table 1) (28).

Some properties of C. difficile and C. sordellii toxins are compared in Table 2. Toxins A and HT exhibit similar physicochemical characteristics. Both toxins are very large with molecular weights of about 300,000 as determined by SDS-PAGE; however, their isoelectric points were slightly different.

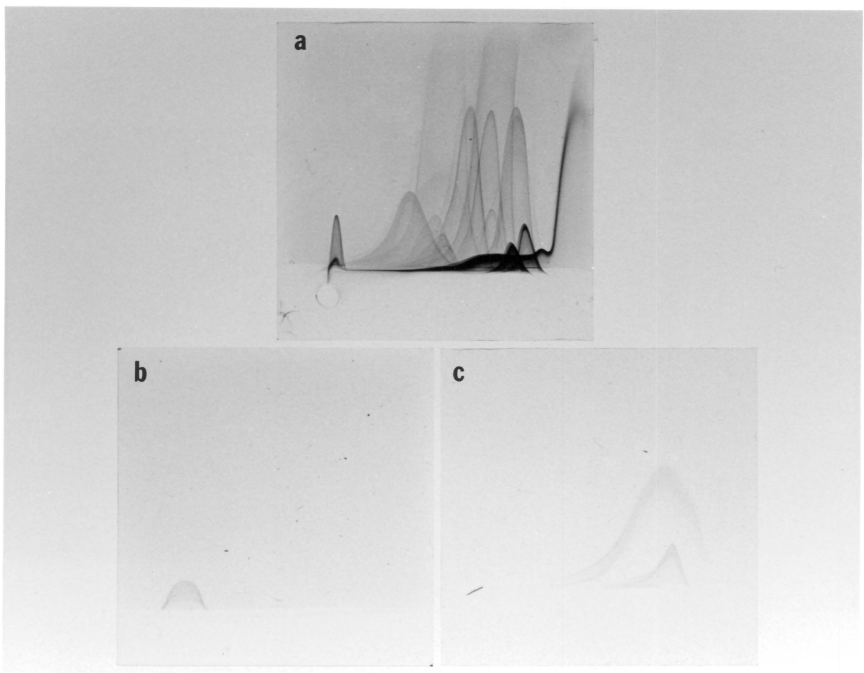

Figure. 1. Analysis of toxin preparations by crossed immunoelectrophoresis. (a) C. sordellii VPI 9048 culture filtrate (75 ug). (b) Toxin HT purified by immunoaffinity chromatography on MAb PCG-4-AffiGel 10 (2.5 ug). (c) Partially purified toxin LT (15 ug). The upper portion of each plate contained 250 ul of rabbit antiserum against culture filtrate from C. sordellii VPI 9048.

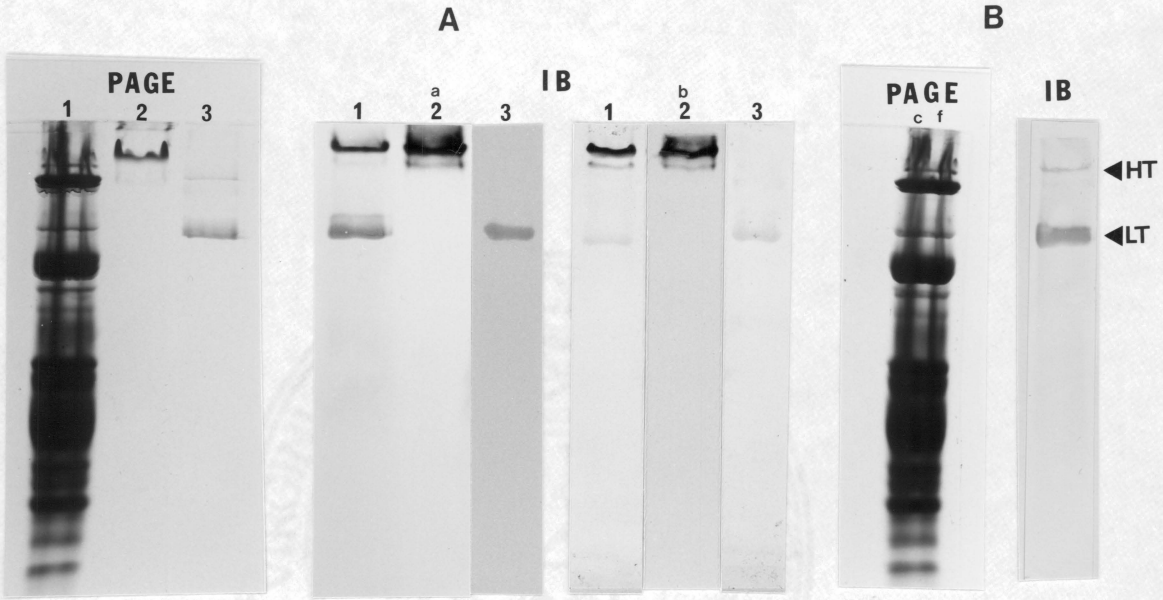

Figure 2. (A) Analysis by PAGE, and immunoblots of C. sordellii VPI 9048 culture filtrate (200 ug) (lanes 1), purified HT (10 ug) (lanes 2) and partially purified LT (12 ug) (lanes 3). PAGE was performed in 4% stacking-7.5% separating gels. After electrophoresis, the proteins were either stained with Coomassie brillant blue R-250 or blotted onto nitrocellulose for immunoblot analysis with (a) affinity-purified toxin A antibodies or (b) MAb G-2. (B) PAGE and immunoblot analysis of C. sordellii culture filtrate (c.f., 200 ug) using the affinity-purified toxin B antibodies. Arrows point to the HT and LT protein bands.

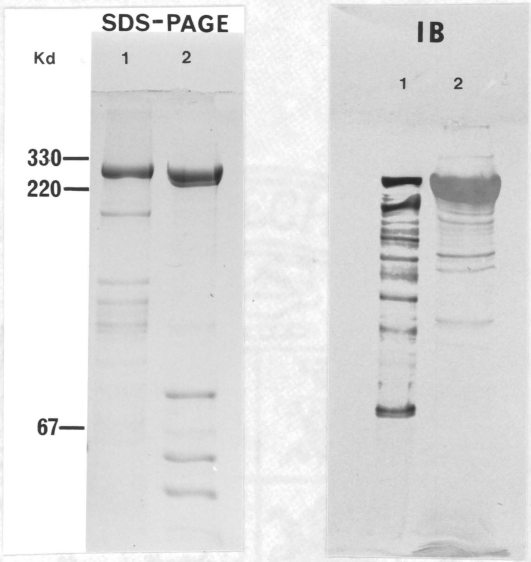

Figure 3. Analysis by SDS-PAGE and immunoblot of purified HT (10 ug) (lanes 1) and partially purified LT (12 ug) (lanes 2). After electrophoresis, the proteins were either stained with Coomassie brillant blue R-250 or blotted onto nitrocellulose for immunoblot analysis with affinity-purified antibodies to toxin A. Kd, kilodaltons.

Table 1. Amino-terminal sequence of toxins A, B, HT and LT.

Toxin	Amino-terminal sequence
	1 5 10 15
A[a]	Ser-Leu-Ile-Ser-Lys-Glu-Glu-Leu-Ile-Lys-Leu-Ala-Tyr-Ser-Ile-Arg-Pro
HT	Ser-Leu-Ile-Ser-Lys-Ser-Glu-Leu-Ile-Lys-Leu-Ala-Tyr-Ser-Ile-Lys-Pro
B[b]	Trp-Leu-Val-Asn-Arg-Lys-Gln-Leu-Glu-Lys-Met-Ala-Asn-Val-Arg-Phe-Arg
LT	Arg-Leu-Val-Asn-Lys-Ala-Gln-Leu-Gln-Lys-Met-Ala-Tyr-Val-Lys-Phe-Asp

[a]Deduced from the nucleotide sequence (C.H. Dove et al., submitted for publication).

[b]Sequence reported by Meador and Tweten (28).

Table 2. Comparison of properties of toxins A, B, HT and LT

Toxin	Native M.W.	Denatured M.W.	pI	pH stability	Heat stability
A[a]	550,000	300,000 (308,103)	5.2-5.7	Stable at pH 4.0 to 10.0	Stable at -20 to 37°C inactivated at 56°C.
HT[b]	525,000	300,000	6.1	Stable at pH 4.0 to 10.0	Stable at -20 to 37°C, inactivated at 56°C.
B[c]	360,000	250,000-300,000	4.1-4.5	Inactivated at pH 4.0 and 10.0	Stable at -20 to 37°C, inactivated at 56°C.
LT[d]	250,000	240,000-260,000	4.5	Stable at pH 5.0 to 8.5	Inactivated at 50°C.

[a]Toxin A was purified by sequential ammonium sulfate precipitation, ion exchange chromatography, and precipitation at pH 5.6 (23, 39). The calculated molecular weight deduced from the nucleotide sequence of toxin A (Dove et al., submitted for publication), is shown in parentheses.

[b]Toxin HT purified by immunoaffinity chromatography on PCG-4 MAb-Affi-Gel 10 (26).

[c]Toxin B was purified by sequential ammonium sulfate precipitation, ion exchange chromatography, and immunoadsorption (23, 39).

[d]Reported by Popoff (30).

Toxins B and LT also have similar properties. Like toxins A and HT, these toxins are extremely large, but are more sensitive to pH extremes (Table 2).

Immunodiffusion analysis with the affinity-purified antibodies to toxin A revealed a reaction of partial identity between toxins A and HT. Likewise, affinity-purified antibodies to toxin B reacted with toxin LT and formed a precipitin line that showed partial identity with toxin B (Fig. 4). The toxins were not immunoprecipitated by the heterologous antiserum (not shown).

Immunoblot analyses. Under nondenaturing conditions the affinity-purified antibodies to toxin A, and the MAb G-2 reacted with the affinity-purified toxin HT, and with HT in the culture filtrate (Fig. 2). Each of these antibody preparations also reacted with the major and minor bands of HT observed by SDS-PAGE as shown with the affinity-purified antibodies to toxin A (Fig. 3). The affinity purified-antibody to toxin A and the MAb G-2 also reacted with toxin LT under non-denaturing (Fig. 2) and denaturing conditions (Fig. 3). MAb G-2 has been shown to react with C. difficile toxins A and B (21). We previously reported that the MAb PCG-4 reacts only with toxin A and with toxin HT (21, 26).

Affinity-purified antibodies to toxin B reacted with the bands corresponding to toxins HT and LT in the culture

filtrate of the toxigenic strain of C. sordellii (Fig. 2). The antibodies to toxins A and B did not react with any antigen in the culture filtrates from the nontoxigenic strains (VPI 2013 and 7319) of C. sordellii.

Amino acid analysis. The amino acid composition of HT is presented in Table 3, and compared with those previously published for toxins A, B, and LT (21, 23, 30).

Biological activity. Immunoaffinity purification of toxin HT resulted in partial inactivation. However, using an HT-ELISA we were able to quantify HT in the partially purified preparation, and determine the specific activities of the native toxin HT. Toxins HT and LT had specific activities in the tissue culture assay of 6.6×10^4 CU/mg and 6.2×10^5 CU/mg, respectively. The specific activities of the toxins in the mouse lethality assay were 1.3×10^4 LD_{100}/mg for HT and 2.0×10^5 LD_{100}/mg for LT. A comparison of the biological activities of the C. difficile and C. sordellii toxins is presented in Table 4.

Affinity-purified antibody to toxin A neutralized the cytotoxicity, lethality for mice and enterotoxicity of toxin HT, but not the biological activities of the partially purified toxin LT. Affinity-purified antibody to toxin B neutralized the cytotoxic and lethal activities of toxin LT

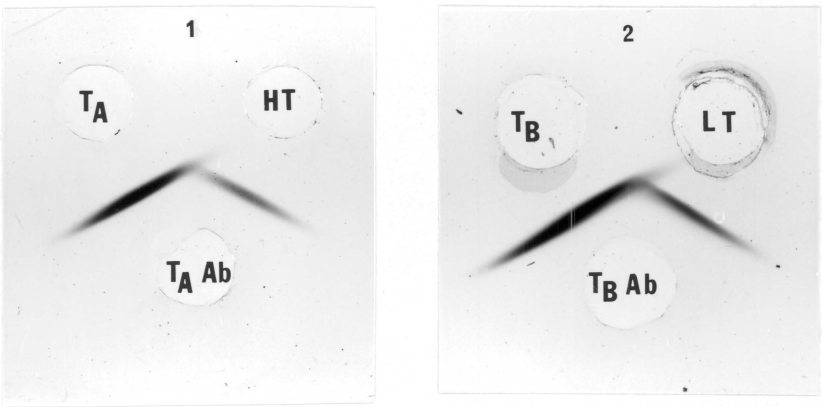

Figure 4. Ouchterlony immunodiffusion analysis. (1) Toxin A (T_A, 20 ug) and HT (20 ug) vs affinity-purified antibodies to toxin A (T_A Ab). (2) Toxin B (T_B, 30 ug) and LT (30 ug) vs affinity-purified antibodies to toxin B (T_B Ab).

Table 3. Amino acid composition of toxins A, B, HT and LT.

Amino acids	mol%			
	T_A[a]	HT[a]	T_B[b]	LT[c]
Asp/Asn	15.0	7.8	14.7	13.4
Thr	5.1	7.6	5.1	5.9
Ser	7.6	8.7	7.5	6.0
Glu/Gln	8.9	6.6	11.5	10.3
Pro	2.1	3.7	2.6	2.9
Gly	7.6	7.3	10.7	8.3
Ala	5.9	5.2	4.5	7.3
Val	5.4	4.5	6.0	6.1
Met	1.0	1.8	0.3	2.5
Ile	8.4	9.8	8.6	7.9
Leu	8.7	10.4	8.0	6.7
Tyr	4.4	5.8	2.8	4.5
Phe	5.4	6.8	5.0	4.9
His	1.8	1.9	1.4	1.8
Lys	8.7	9.0	6.4	6.9
Arg	3.1	2.7	2.5	2.7
Trp	1.2	1.5	1.9	ND
Cys	0.8	ND	0.5	1.3

[a]Toxins A and HT were purified by immunoaffinity chromatography onPCG-4 antibody Affi-Gel 10 (21, 26).

[b]Reported by Lyerly et al (23).

[c]Reported by Popoff (30).

ND - not determined

Table 4. Biological activities of toxins A, B, HT, and LT.

Toxin	Tissue culture dose	Lethal dose in mice	Enterotoxic dose in rabbit ileal loop
A[a]	10 ng	50 - 90 ng	1 ug
HT[b]	15 ng	75 ng	2 ug
B[a]	0.2-1.0 pg	50 ng	Negative
LT[c]	1.6 ng	5 ng	Negative

[a]Lyerly et al (23) and Sullivan et al (39).

[b]Doses are based on the amount of toxin HT in a partially purified preparation as determined by ELISA (25, 26).

[c]Popoff reported values of 16 and 2.5 ng for TCD and LD, respectively. A low, non-hemorhagic enterotoxic response in guinea pig intestinal loops was also reported (30).

but did not neutralize the biological activities of HT. None of the individual highly purified antibodies to either toxins A or B totally neutralized the cytotoxic or lethal activities of the C. sordellii culture filtrate that contained both toxins. However, a combination of toxin A and toxin B antibodies did neutralize the cytotoxicity and lethality of the culture filtrate, indicating that the toxicity of the culture filtrate of C. sordellii is due to the toxins HT and LT. Antibody to toxin A (MAb PCG-4 and the affinity-purified antibody) neutralized all of the enterotoxic activity of C. sordellii culture filtrate.

DISCUSSION

The approach we used to obtain a partially purified preparation of toxin LT was similar to that used for the purification of C. difficile toxin B (39). The anion exchange step allowed for the separation of toxins HT from the LT and adsorption of HT with the PCG-4 MAb-gel further guaranteed a complete removal of HT from the LT preparation. In addition, the cytotoxic and lethal activities of this preparation were completely neutralized by antibody to toxin B.

Toxins HT and A have molecular weights in excess of 300,000 as determined by SDS-PAGE (21, 26). The gene for toxin A has been sequenced, and encodes a polypeptide with a

calculated molecular weight of 308,103 (Dove et al., submitted for publication), which supports the observations from SDS-PAGE. Toxins LT and B are also extremely large proteins with molecular weights in excess of 250,000 (21, 28, 30). Faster-migrating bands are observed when toxins HT and LT are denatured and analyzed by SDS-PAGE. These minor bands react with highly specific polyclonal antibodies to either toxin A or toxin B in immunoblots. The minor bands generated from toxin HT also have been shown to react with the MAb PCG-4, but do not react with antibodies to non-toxigenic strains (26). The appearance of similar minor bands also is a characteristic of toxins A and B of C. difficile.

Since differences in the pI's of toxins A and HT have been demonstrated, it was of interest to compare the amino acid composition of each toxin. Comparison of amino acid composition revealed two-fold difference in the levels of aspartic acid/asparagine, and minor differences in the levels of other amino acid residues, which may explain the difference in pI. However, the two toxins had similar specific activities (Table. 4), indicating that despite the compositional differences the biological activities were not significantly changed.

High levels of homology were observed between the amino-terminal sequences of toxins A and HT. The amino-terminal

region of LT also bears close homology with the recently published sequence for toxin B (28). The fact that most of the amino acid changes observed would involve a single nucleotide change, further supports the fact that these toxins are highly related.

Affinity-purified toxin HT retained its biological activities, which are similar to those observed with toxin A and included cytotoxicity (cell rounding), lethality in mice, and enterotoxicity. However, elution of HT from the antibody column with $MgCl_2$ resulted in partial inactivation of the toxin. Nevertheless, we were able to quantitate HT in a partially purified preparation by an HT-ELISA, and thus estimate its specific activities in its native state. Using this approach, we found that the specific activities of toxin HT in the biological assays are very similar to those of toxin A (Table. 4).

As with toxin B, toxin LT was found to be cytotoxic and lethal but not enterotoxic. However, significant differences were observed in the cytotoxic titers of these toxins. Toxin B is at least 1000-fold more cytotoxic than LT.

Antibodies to either toxin A or toxin B alone did not neutralize all of the cytotoxic or lethal activities of the C. sordellii culture filtrate. However, antibodies to both toxins or antiserum to C. difficile completely neutralized these activities. This indicates that toxins HT and LT are

responsible for all the cytotoxic and lethal activities of the C. sordellii culture filtrate. Antibodies to toxin A as well as the MAb PCG-4, completely neutralized the enterotoxicity of the C. sordellii culture filtrate, indicating that this activity is due solely to toxin HT, just as the enterotoxicity of the C. difficile cultures filtrate is due to toxin A (24).

We attempted to purify toxin HT by use of receptor-affinity column procedure that has been described for the purification of toxin A. In this procedure, toxin A binds to the trisaccharide receptor Galα1-3Galβ1-4GlcNAc on bovine thyroglobulin at 4^{o}C, and then is eluted by increasing the temperature to 37^{o}C (14). Although this approach resulted in partial purification of toxin HT, the final product was not homogeneous by either PAGE or crossed-immunoelectrophoresis. The results did show that toxin HT did bind to this trisaccharide. Further evidence indicating that toxins A and HT share similar receptor-binding sites came from neutralization experiments with the MAb PCG-4. This MAb neutralizes the enterotoxic activities of toxin HT and toxin A (21, 26), and has been shown to react with a peptide representing the region of toxin A that binds to the trisaccharide Galα1-3Galβ1-4GlcNAc (31). Taken together, these results as well as the work of Price et al. (31), indicates that both toxins have similar receptor-binding

sites.

No precipitation of either HT or LT was observed in the Ouchterlony-double immunodiffusion test with the heterologous antibody preparation, and cross-neutralization experiments showed that the antigenic determinants involved in neutralization are not shared by these toxins. Nevertheless, the lack of cross-neutralization or cross-reactivity by immunodiffusion assays does not preclude the presence of common antigenic determinants between these toxins. The more sensitive immunoblot assay did show some cross-reactivity. Affinity-purified antibody to toxin A reacted strongly with HT and also showed some reactivity with LT. The intensity of the reaction of the MAb G-2 was also higher for HT than for LT. Likewise, affinity-purified antibody to toxin B showed a strong reaction with toxin LT but did react weakly with HT. Therefore, the data supports the presence of shared antigenic determinants between toxins HT and LT, but indicates a lower binding affinity of the antibodies to cross-reactive epitopes in the heterologous toxin. The MAb PCG-4, which appears to attach to the receptor binding region of toxin A, reacted only with HT.

Immunological cross-reactivity between toxins produced by other organisms has been previously documented. The enterotoxins produced by Staphylococcus aureus comprise a group of proteins that once were regarded as antigenically

distinct. However, it now has been shown that varying degrees of immunological relatedness exist between these toxins (36). This type of phenomenon is also observed with the shiga-like toxins produced by some strains of Escherichia coli. These toxins have been designated shiga-like toxin I (SLT-I) and shiga-like toxin II (SLT-II) and they have similar biological activities and physicochemical properties (38). In addition, SLT-I and SLT-II share short regions of high amino acid sequence homology (70-100%) with an overall homology of 56%, however, the toxins are not cross-neutralized by heterologous antiserum (13, 38).

As with the shiga-like toxins, C. difficile toxins A and B are not cross-neutralized by heterologous antiserum (18); however, comparison of the deduced amino acid sequence of toxins A and B revealed regions of homology between these toxins (J.L. Johnson, manuscript in preparation) which further supports our observations with the toxins of C. sordellii.

Although the four toxins have some similarities in physicochemical and biological properties, only toxins A and HT were enterotoxic. One can speculate that homologous regions shared by these toxins are required for their similarities. Future elucidation of the genetic and structural details of these toxins might help to explain these relationships.

In conclusion, the findings presented herein support previous observations regarding the relationship between the toxins of C. difficile and the toxins of C. sordellii, and demonstrate greater divergence between toxins B and LT than between toxins A and HT. Further study of these antigenic and functional differences may provide a better understanding of these toxins.

LITERATURE CITED

1. **Allo, M., J. Silva. R. Fekety, G.D. Rifkin, and H. Waskin.** 1979. Prevention of clindamycin-induced colitis in hamsters by Clostridium sordellii antitoxin. Gastroenterology 76:351-355.

2. **Al Mashat, R.R., and D.J. Taylor.** 1983. Bacteria in enteric lession in cattle. Vet. Rec. 112:19.

3. **Arseculeratne, S.N., R.G. Pannabokke, and S. Wijesundera.** 1969. The toxins responsible for the lesions of Clostridium sordellii gas gangrene. J. Med. Microbiol. 2:37-53.

4. **Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of Clostridium difficile causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. 2:629-635.

5. **Barlett, J.G., T.-W. Chang, M. Gurwith, S.L. Gorbach, and A.B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin producing clostridia. N. Engl. J. Med. 298: 531-534.

6. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

7. **Chang, T.-W., S.L. Gorbach, and J.G. Bartlett.** 1978. Neutralization of Clostridium difficile toxin by Clostridium sordellii antitoxins. Infect. Immun. 22:418-422.

8. **Edelhoch, H.** 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry 6:1948-1954.

9. **Ehrich, M., R.L. Van Tassell, J.M. Libby, and T.D. Wilkins.** 1980. Production of Clostridium difficile antitoxin. Infect. Immun. 28:1041-1043.

10. **George, R.H., J.M. Symonds, F. Dimock, J.D. Brown, Y. Arabi, N. Shinagawa, M.R.B. Keighley, J. Alexander-Williams, and D.W. Burdon.** 1978. Identification of Clostridium difficile as a cause of pseudomembranous colitis. Br. Med. J. 1:695.

11. **Hogan, S.F., and K. Ireland.** 1989. Fatal acute

endometritis resulting from Clostridium sordellii. Am. J. Clin. Pathol. 91:104-106.

12. **Holdeman, L.V., E.P. Cato, and W.E.C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.

13. **Jackson, M.P., R.J. Neill, A.D. O'Brien, R.K. Holmes, and J.W. Newland.** 1987. Nucleotide sequence analysis and comparison of the structural genes for shiga-like toxin I and shiga-like toxin II encoded by bacteriophages from Escherichia coli 933. FEMS Microbiol. Lett. 44:109-114.

14. **Krivan, H.C., and T.D. Wilkins.** 1987. Purification of Clostridium difficile toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. 55:1873-1877.

15. **Laemmli, U.K.** 1970. Cleavage of Structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) 227:680-685.

16. **Larson, H.E., and A.B. Price.** 1977. Pseudomembranous colitis: presence of clostridial toxin. Lancet ii:1312-1314.

17. **Libby, J.M., B.S. Jortner, and T.D. Wilkins.** 1982. Effects of the two toxins of Clostridium difficile in antibiotic-associated cecitis in hamsters. Infect. Immun. 36:822-829.

18. **Libby, J.M., and T.D. Wilkins.** 1982. Production of antitoxins to two toxins of Clostridium difficile and immunological comparison of the toxins by cross-neutralization studies. Infect. Immun. 35:374-376.

19. **Lyerly, D.M., H.C. Krivan, and T.D. Wilkins.** 1988. Clostridium difficile: its disease and toxins. Clin. Microbiol. Rev. 1:1-18.

20. **Lyerly, D.M., D.E. Lockwood, S.H. Richardson, and T.D. Wilkins.** 1982. Biological activities of toxins A and B of Clostridium difficile. Infect. Immun. 35:1147-1150.

21. **Lyerly, D.M., C.J. Phelps, J. Toth, and T.D. Wilkins.** 1986. Characterization of toxins A and B of Clostridium difficile with monoclonal antibodies. Infect. Immun. 54:70-76.

22. **Lyerly, D.M., C.J. Phelps, and T.D. Wilkins.** 1984. Monoclonal and specific polyclonal antibodies for

immunoassay of Clostridium difficile toxin A. J. Clin. Microbiol. 21:12-14.

23. **Lyerly, D.M., M.D. Roberts, C.J. Phelps, and T.D. Wilkins.** 1986. Purification and properties of toxins A and B of Clostridium difficile. FEMS Microbiol. Lett. 33:31-35.

24. **Lyerly, D.M., K.E. Saum, D.K. MacDonald, and T.D. Wilkins.** 1985. Effects of Clostridium difficile toxins given intragastrically to animals. Infect. Immun. 47:349-352.

25. **Lyerly, D.M., N.M. Sullivan. and T.D. Wilkins.** 1983. Enzyme-lynked immunosorbent assay for Clostridium difficile toxin A. J. Clin. Microbiol. 17:72-78.

26. **Martinez, R.D., and T.D. Wilkins.** 1988. Purification of Clostridium sordellii hemorrhagic toxin and cross-reactivity with Clostridium difficile toxin A (enterotoxin). Infect. Immun. 56:1215-1221.

27. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262:10035-10038.

28. **Meador III, J., and R.K. Tweten.** 1988. Purification and characterization of toxin B from Clostridium difficile. Infect. Immun. 56:1708-1714.

29. **Popoff, M.R.** 1984. Bacteriological examination in enterotoxaemia of sheep and lamb. Vet. Rec. 114:324.

30. **Popoff, M.R.** 1987. Purification and characterization of Clostridium sordellii lethal toxin and cross-reactivity with Clostridium difficile cytotoxin. Infect. Immun. 55:35-43.

31. **Price, S.B., C.J. Phelps, T.D. Wilkins, and J.L. Johnson.** 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of Clostridium difficile. Curr. Microbiol. 16:55-60.

32. **Richards, S.M.** 1982. Clostridium sordellii in lambs. Vet. Rec. 111:22.

33. **Rifkin, G.D., F.R. Fekety, and J. Silva.** 1978. Neutralization by Clostridium sordellii antitoxin of toxins implicated in clindamycin-induced cecitis in hamsters. Gastroenterology. 75:422-424.

34. **Rifkin, G.D., F.R. Fekety, J. Silva, and R.B. Sack.**

1977. Antibiotic-induced colitis. Implication of a toxin neutralised by Clostridium sordellii antitoxin. Lancet ii:1103-1106.

35. Spackman, D.H., W.H. Stein, and S. Moore. 1958. Automatic recording apparatus for the use in the chromatography of amino acids. Anal. Chem. 30:1190-1206.

36. Spero, L., B.A. Morlock, and J.F. Metzger. 1978. On the cross-reactivity of staphylococcal enterotoxins A, B, and C. J. Immunol. 120:86-89.

37. Sterne, M., and L.M. Wentzel. 1950. A new method for the large scale production of high-titre botulinum formol-toxoid types C and D. J. Immunol. 35:1032-1040.

38. Strockbine, N.A., L.M. Marques, J.W. Newland, H.W. Smith, R.K. Holmes, and A.D. O'Brien. 1986. Two toxin-converting phages from Escherichia coli 0157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect. Immun. 53:135-140.

39. Sullivan, N.M., S. Pellett, and T.D. Wilkins. 1982. Purification and characterization of toxins A and B of Clostridium difficile. Infect. Immun. 35:1032-1040.

40. Taylor, N.S., G.M. Thorne, and J.G. Barlett. 1981. Comparison of two toxins produced by Clostridium difficile. Infect. Immun. 34:1036-1043.

41. Yamakawa, K., S. Nakamura and S. Nishida. 1985. Separation of two cytotoxins of Clostridium sordellii strains. Microbiol. Immunol. 29:553-557.

Section V. Overall discussion and summary.

Clostridium sordellii and Clostridium difficile were isolated in 1922 by Sordelli and 1935 by Hall and O'Toole, respectively (14, 36). The toxigenic nature of each organism was immediately noticed by these investigators; however, the antigenic cross-reactivity between the toxins produced by the organisms was not recognized until the late 1970's (3, 6, 8, 12, 33, 34). Shortly after this, the toxins of C. difficile were purified and much information has been gained about the properties of these toxins and their role in disease (4, 5, 20, 21, 22, 23, 24, 25, 37, 38). However, very little information was obtained about the related toxins produced by C. sordellii. The main purpose of my study was to obtain a better understanding of the toxins of C. sordellii and to compare their properties with those of toxins A and B of C. difficile.

I purified and characterized toxin HT of C. sordellii and show that this toxin is related to the toxin A (enterotoxin) of C. difficile. In addition to the antigenic relationship between these toxins, I demonstrated that their biological and physicochemical properties are very similar.

Most investigators agree that toxins A and B are very large proteins; however, since their molecular size is unusually large questions were still being raised about the primary structure of these toxins. I found that, like

toxin A, toxin HT is also a large protein with a molecular weight of about 300,000, and that toxin LT is also a large protein with a molecular weight in excess of 250,000. These findings, together with the work of Dr. Johnson and coworkers, corroborates the information obtained with the toxins of C. difficile, and helps to confirm that these toxins are indeed very large.

My work, as well as the work of Meador and Tweeton (29), and Dove et al., (submitted for publication) further demonstrated the similarities and relationship of the toxins. Similarities between the amino-terminal sequences of toxins A and HT, and toxins B and LT were shown, which corroborates the fact that we are dealing with similar toxins. It is worth mentioning that these findings came from independent research groups, and that different technical approaches were used (see section IV for details).

The major physicochemical difference between toxins A and HT are their isoelectric points. As discussed in section IV, this could be explained by their differences in amino acid composition. These differences could affect their surface charges conferring different isoelectric points.

One of the most interesting properties of toxin A is its enterotoxic activity. Like toxin A, I found that toxin HT is enterotoxic, and elicits the accumulation of an hemorrhagic fluid in the rabbit ileal loop (27). I also demonstrated

that all the enterotoxic activity in the culture filtrate of C. sordellii is due to toxin HT. This situation is analogous to that seen with cultures filtrates of C. difficile, in which toxin A is responsible for all of the enterotoxicity (24).

Toxin A binds to the trisaccharide Galα1-3Galβ1-4GlcNAc, which has been shown to function as a receptor for toxin A (17, 18, 39). The information that I obtained indicates that HT also binds to this carbohydrate sequence; however, this toxin seems to bind with less avidity. Efforts to purify HT by the bovine-thyroglobulin method were unsuccessful. The small amount of toxin retained in the column was contaminated with thyroglobulin, probably resulting from cleavage by proteases present in the culture filtrate. Nevertheless, other evidence indicates that toxins A and HT have very similar receptor-binding sites and therefore their receptors may be very similar, too. Some of this evidence came from studies with MAb PCG-4. This monoclonal antibody has been shown to bind to (or to bind a site close to) the receptor binding region of toxin A (32). MAb PCG-4 also binds HT; however, I demonstrated that the strength of the binding is lower when compared to toxin A. The epitope recognized by PCG-4 is therefore also present in HT; however, this epitope or the region near it could be slightly different than the homologous region on toxin A.

Thus, it is possible that the receptor binding site of HT may be structurally different from that of toxin A, which may explain the differences in binding to the trisaccharide receptor. A similar phenomenon was observed with the shiga like toxin II (SLT-II) and shiga like toxin II variant (SLT-IIv) produced by some strains of Escherichia coli. These toxins share 79% amino acid sequence identity in their binding subunits; however, SLT-IIv does not bind to the carbohydrate-receptor that SLT-II binds (41). Additional evidence indicating that toxins A and HT have similar receptors came from cytotoxicity studies with the F-9 cell line. Compared to the Chinese Hamster Ovary-K9 (CHO-K9) cell line, the F-9 is about a 100-fold more sensitive to toxin A (39). The increased sensitivity is apparently due to the expression on the cell surface of a glycoconjugate containing the trisaccharide that serves as a receptor for toxin A. The F-9 cell line also has an increased sensitivity to HT of about 100 to 120-fold as compared to the CHO-K9 cells, which again suggests that the receptor for both toxins may be very similar.

While the work described in this dissertation was in progress, Popoff published on the purification and characterization of toxin LT (31). Most of my findings are similar to his observations; however, my results with respect to the inactivation of the toxin with trypsin

disagree with those of Popoff (31). He indicated that LT is considerably resistant to treatment with these protease; however, my results indicate that LT is readily inactivated by treatment with this enzyme (Martinez, unpublished). I found that LT is very unstable and loses biological activity even after storage at 4°C. One of the main differences between toxins B and LT is in their cytotoxic doses, with toxin B being about a 1000-fold more cytotoxic in the CHO-K9 assay. This difference in cytotoxicity may result from partial inactivation of the toxin LT by proteases produced by the organism, since C. sordellii is more proteolytic than C. difficile. Alternately, LT may not be as active as toxin B on tissue-cultured cells because of differences in binding and/or intracellular processing.

Popoff (31) reported that toxin LT induces a low, non-hemorrhagic fluid accumulation when injected into guinea pig ileal-loops. Contrary to this, my results showed that injection of LT into rabbit ileal-loops does not induce fluid accumulation. It is possible that guinea pigs are more sensitive to the action of toxin LT in this assay. LT induces vascular permeability in skin assays, which may explain that LT can exert some sort of fluid accumulation.

All C. difficile strains that have been tested either produce both toxins or neither toxin (19, 25), suggesting that expression of the toxins is coregulated, and I observed

a similar phenomenon with the toxins of C. sordellii. Yamakawa et al., reported that one strain of C. sordellii produces toxin LT, but not HT (43). However, they used rabbit ileal-loops as the detection method for HT, and this method is not sensitive enough to detect small amounts of the toxin. I tried to obtain this strain as well as a strain that has been reported to produce large amounts of these toxins, but these investigators refused my request. A high toxin producer would have facilitated my research since most strains of C. sordellii produce low amounts of toxins, which combined with the presence of proteolytic activity in the culture filtrate makes purification by classical techniques extremely difficult.

Previous studies indicated that the toxins produced by C. difficile and C. sordellii are not identical (3, 8, 12). Studies described in this dissertation (amino acid composition, antibodies binding studies and immunodiffusion analyses) confirmed these observations and revealed that each toxin has unique epitopes. I determined if antibodies against these unique epitopes have any role in neutralization of cytotoxicity of toxins A and HT. Specific and common antibodies to toxins A and HT were isolated from anti-A and anti-HT polyclonal antibodies by affinity chromatography. Anti-A and anti-HT antibodies were passed through a column containing an immobilized cross-reacting

toxin. Antibodies that bound to both toxins were designated as common-antibodies, and recognized epitopes shared by both toxins. Antibodies that were not adsorbed on the toxin-column were designated as specific antibodies since they reacted only with the homologous toxin as determined by immunoassays. Antibodies common to both toxins neutralized the cytotoxic activity of toxins A and HT. On the other hand, specific antibodies did not neutralize the cytotoxic activity of either toxin, which suggests that the epitopes recognized for neutralization of toxic activity are shared by these toxins. This observation is in agreement with the finding that the monoclonal antibody PCG-4 neutralizes the enterotoxic activity of both toxins, and indicates that although the toxins have some distinct epitopes the regions involved in toxic activity remain highly conserved.

Sporulation and production of toxin HT. Several spore-forming bacteria produce protein toxins of medical and economic importance. Several subspecies of Bacillus thuringiensis, for example, produce entomocidal toxins which are synthesized during the sporulation process and are contained primarily as a parasporal crystalline inclusions (1, 7). An example in the medical aspect is the enterotoxin of Clostridium perfringens. Of several toxins produced by this organism, the enterotoxin is the only one that is synthesized by sporulating cells. This toxin is a spore coat

structural component (10, 11, 13), and is responsible for one of the most common types of food poisoning in the United States (28).

The association of sporulation and the production of toxin HT by C. sordellii was suggested by Arseculeratne et al. (2) and, therefore, it was of interest to test this observation. To induce sporulation cultures of C. sordellii were grown in a medium containing 1% peptone, 0.5% yeast, and 0.5% pepticase. The pH was adjusted to 8.0 and the medium was kept anaerobic under nitrogen. After the cells were grown for 48 h at 37°C with slight agitation, the cultures were examined for the presence of spores. Free spore preparations were obtained and proteins were extracted by procedures described previously (9, 13, 40). The extracted proteins were separated on SDS-PAGE, electroblotted onto nitrocellulose, and incubated with the MAb PCG-4 or affinity purified antibody to toxin A to detect any HT or HT-like protein. The results of these experiments were unfruitful since I found no evidence that toxin HT is associated to the spores. It is my experience with C. sordellii that the toxin remains in the culture supernatant, and that there is no significant sporulation when the cells are grown for the purpose of toxin purification.

Plasmid studies. The role of plasmids in the expression of virulence factors has been an area of interest, and it has

become increasingly evident that these extrachromosomal elements often play an important role in the pathogenic potential of a variety of microorganisms. For instance, plasmids in a variety of bacteria have been shown to specify virulence determinants including among others, invasiveness, resistance to antimicrobial compounds, as well as toxin production (26). Sharing of functional and structural features between toxins (or other proteins) is very likely indicative of evolution from a common ancestral gene. Since many toxin genes are located on plasmids, this may conceivably represent a genetic mechanism for the presence of similar toxins in different bacterial species. I investigated the possibility that the toxins HT and LT of C. sordellii are plasmid encoded; however, my attempts were unsuccessful. The presence of nucleases as well as the presence of a polysaccharide material were a hindrance for the isolation of plasmids. I also attacked the problem indirectly by trying to cure C. sordellii from putative plasmid(s) related to toxin production. I used growth at high temperature and/or growth in the presence of different chemicals (i.e., ethidium bromide, acridine orange, or novobiocin). However, these results were also negative.

Several workers have described the physical isolation of large plasmids from C. difficile; however, these plasmids are present in both toxigenic and nontoxigenic strains, and

do not appear to be involved in toxin production (15, 30, 42). Schallehn (35) identified bacteriophages that are active in both C. difficile and C. sordellii, but she found no evidence for involvement of the bacteriophages in toxin production. More recent experiments by Johnson et al. suggest that the gene for toxin A is located on the chromosome (16). The information that I obtained tends to indicates that the same is true for the genes coding for the toxins of C. sordellii; however, a role for plasmids in toxin production cannot be completely ruled out at this moment.

Future directions. The work described herein has answered several questions on the relationship of the toxins HT and LT of C. sordellii to the toxins A and B of C. difficile, but obviously, several other areas of research remain to be explored.

Cloning and sequencing of the genes coding for the toxins of C. sordellii should provide a more detailed view of the differences between these toxins and those of C. difficile. Since the toxins of C. difficile have been cloned, fragment(s) of the genes for these toxins could be used to screen colonies resulting from shotgun cloning for the isolation of C. sordellii toxin genes.

Comparison of the nucleotide sequences of the toxins may provide an idea of the evolution and ancestry of the

toxins. For example, the G + C content could be determined and examined for similarities with others genes of either C. difficile or C. sordellii.

Once the binding region of toxin A is described in detail, it could be compared with the homologous region in HT. Detailed comparison of these regions may provide some insight on the difference in binding efficiency to the trisaccharide Galα1-Galβ1-4GlcNAc. These studies could be very helpful in determining how proteins interact with carbohydrates, and specifically, what amino acids are involved.

Mapping and comparison of the active sites of these toxins should provide valuable information about the differences in cytotoxic activity between toxins B and LT. It will be interesting to determine whether this difference is due to loss of enzymatic activity by LT or because this toxin uses a receptor different from that of toxin B.

Important questions are still pending on how cells regulate the production of these large proteins. Are the regulatory mechanisms the same for the toxins of C. difficile as for the toxins of C. sordellii? Answers to these questions may provide important insight on how C. difficile and C. sordellii cause disease.

The project described in this dissertation represents the first comprehensive study comparing the toxins HT and LT of

C. sordellii to the toxins A and B produced by C. difficile.

I hope that the information contained in it will be helpful in future investigations.

LITERATURE CITED.

1. **Andrews, R.E., Jr., and L.A. Bulla, Jr.** 1980. Toxins of sporeforming bacteria, p. 57-63. In H.S. Levinson, A.L. Sonenshein, and D.P. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.

2. **Arseculeratne, S.N., R.G. Panabokke, and S. Wijensundera.** 1969. The toxins responsible for the lesions of Clostridium sordellii gas gangrene. J. Med. Microbiol. 2:37-53.

3. **Aswell, J.E., M. Ehrich, R. Van Tassell, G.C. Tsai, L.V. Holdeman, and T.D. Wilkins.** 1979. Characterization and comparison of Clostridium difficile and other clostridial toxins. In D. Schlessinger (ed.) Microbiology-1979. American Society for Microbiology, p. 272-275, Washington, D.C.

4. **Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Ueno, and Y. Nozawa.** 1984. Biochemical characterization and biologic actions of two toxins (D-1 and D-2) from Clostridium difficile. Rev. Infect. Dis. 6:S11-S20.

5. **Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of Clostridium difficile causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. 2:629-635.

6. **Bartlett, J.G., T.-W. Chang, M. Gurwith, S.L. Gorbach, and A.B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin producing clostridia. New. Engl. J. Med. 298:531-534.

7. **Bulla, L.A., Jr., and A.A. Yousten.** 1979. Bacterial insecticides, p. 91-114. In A.H. Rose (ed.), Economic microbiology, vol. 4. Academic Press, New York.

8. **Chang, T.-W., S.L. Gorbach, and J.G. Bartlett.** 1978. Neutralization of Clostridium difficile toxin by Clostridium sordellii antitoxins. Infect. Immun. 22:418-422.

9. **Deutscher, M.P., and A. Konberg.** 1968. Biochemical studies of bacterial sporulation and germination. J. Biol. Chem. 243:4653-4660.

10. **Duncan, C.L., and D.H. Strong.** 1969. Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of Clostridium perfringens. J. Bacteriol.

100:86-94.

11. **Duncan, C.L., D.H. Strong, and M. Sebald.** 1972. Sporulation and enterotoxin production by mutants of Clostridium perfringens. J. Bacteriol. 110:378-391.

12. **Ehrich, M., R.L. Van Tassell, J.M. Libby, and T.D. Wilkins.** 1980. Production of Clostridium difficile antitoxin. Infect. Immun. 28:1041-1043.

13. **Frieben, W.R., and C.L. Duncan.** 1975. Heterogeneity of enterotoxin-like protein extracted from spores of Clostridium perfringens type A. Eur. J. Biochem. 55:455-463.

14. **Hall, I.C., and E. O'Toole.** 1935. Intestinal flora of new-born infants. Am. J. Dis. Child. 49:390-302.

15. **Hayter, P.M., and J.W. Dale.** 1984. Detection of plasmids in clinical isolates of Clostridium difficile.

16. **Johnson, J.L., C.H. Doves, S.B. Price, T.W. Sickles, C.J. Phelps, and T.D. Wilkins.** 1988. The toxin A gene of Clostridium difficile, p. 115-123. In J.M. Hardie and S.P. Borriello (ed.), Anaerobes Today. John Wiley and sons, New York.

17. **Krivan, H.C., G.F. Clark, D.F. Smith, and T.D. Wilkins.** 1986. Cell surface binding site for Clostridium difficile enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNac. Infect. Immun. 53:573-581.

18. **Krivan, H.C., And T.D. Wilkins.** 1987. Purification of Clostridium difficile toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. 55:1873-1877.

19. **Laughon, B.E., R.P. Viscidi, S.L. Gdovin, R.H. Yolken, and J.G. Bartlett.** 1984. Enzyme immunoassays for detection of Clostridium difficile toxins A and B in fecal specimens. J. Infect. Dis. 149:781-788.

20. **Lyerly, D.M., H.C. Krivan, and T.D. Wilkins.** 1988. Clostridium difficile: its disease and toxins. Clin. Microbiol. Rev. 1:1-18.

21. **Lyerly, D.M., D.E. Lockwood, S.H. Richardson, and T.D. Wilkins.** 1982. Biological activities of toxins A and B of Clostridium difficile. Infect. Immun. 35:1147-1150.

22. **Lyerly, D.M., C.J. Phelps, J. Toth, and T.D. Wilkins.**

1986. Characterization of toxins A and B of Clostridium difficile with monoclonal antibodies. Infect. Immun. 54:70-76.

23. **Lyerly, D.M., M.D. Roberts, C.J. Phelps, and T.D. Wilkins.** 1986. Properties of toxins A and B of Clostridium difficile. FEMS Microbiol. Lett. 33:31-35.

24. **Lyerly, D.M., K.E. Saum, D. McDonald, and T.D. Wilkins.** 1982. Effect of toxins A and B given intragastrically to animals. Infect. Immun. 47:349-352.

25. **Lyerly, D.M., N.M. Sullivan, and T.D. Wilkins.** 1983. Enzyme-linked immunosorbent assay for Clostridium difficile toxin A. J. Clin. Microbiol. 17:72-78.

26. **Maas, W.K.** 1985. Genetic aspects of toxigenesis in bacteria. p. 17-26. In F. Dorner and J. Drews (ed.). Pharmacology of bacterial toxins. Pergamon Press, N.Y.

27. **Martinez, R.D., and T.D. Wilkins.** 1988. Purification and characterization of Clostridium sordellii hemorrhagic toxin and cross-reactivity with Clostridium difficile toxin A (Enterotoxin). Infect. Immun. 56:1215-1221.

28. **McDonel, J.L.** 1985. Toxins of Clostridium perfringens types A, B, C, D, and E. p. 493-501. In F. Dorner and J. Drews(ed.). Pharmacology of bacterial toxins. Pergamon Press, N.Y.

29. **Meador J., III., and R.K. Tweeten.** 1988. Purification and characterization of toxin B from Clostridium difficile. Infect. Immun. 56:1708-1714.

30. **Muldrow, L.L., E.R. Archibald, O.L. Nunez-Montiel, and R.J. Sheehy.** 1982. Survey of the extrachromosomal gene pool of Clostridium difficile. J. Clin. MIcrobiol. 16:637-640.

31. **Popoff, M.R.** 1987. Purification and characterization of of Clostridium sordellii lethal toxin and cross-reactivity with Clostridium difficile cytotoxin. Infect. Immun. 55:35-43.

32. **Price, S.B., C.J. Phelps, T.D. Wilkins, and J.L. Johnson.** 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of Clostridium difficile. Curr. Microbiol. 16:55-60.

33. **Rehg, J.** 1980. Cecal toxin(s) from guinea pigs with

cliyndamycin-associated colitis, neutralized by Clostridium sordellii antitoxin. Infect. Immun. 27:387-390.

34. **Rifkin, G.D., F.R. Fekety, and J. Silva.** 1978. Neutralization by Clostridium sordellii antitoxin of toxins implicated in clindamycin-induced cecitis in hamsters. Gastroenterology. 75:422-424.

35. **Schallehn, G.** 1985. Clostridium sordellii bacteriophage active in Clostridium difficile. Lett. Appl. Microbiol. 1:71-74.

36. **Sordellii, A.** 1922. Un germen anaerobio de las gangrenas gaseosas. Rev. Asoc. Med. Argent. 35:308-310.

37. **Sullivan, N.M., S. Pellett, and T.D. Wilkins.** 1982. Purification and characterization of toxins A and B of Clostridium difficile. Infect. Immun. 35:1032-1040.

38. **Taylor, N.S., G.M. Thorne, and J.G. Bartlett.** 1981. Comparison of two toxins of Clostridium difficile. Infect. Immun. 34:1036-1043.

39. **Tucker, K.D., and T.D. Wilkins.** 1989. Clostridium difficile toxin A uses Galα1-3Galβ1-4GlcNac as a funtional receptor. Absrt. Annu. Meet. Am. Soc. Microbiol, p. 63.

40. **Watabe, K., Y. Kakiuchi, and M. Kondo.** 1975. Solubilization of coat proteins from Bacillus thiaminolyticus spores. Microbios. 12:221-224.

41. **Weinstein, D.L., M.P. Jackson, J.E. Samuel, R.K. Holmes, and A.D. O'Brien.** 1988. Cloning and sequencing of a shiga-like toxin type II variant from an Escherichia coli strain responsible for edema disease of swine. J. Bacteriol. 170:4223-4230.

42. **Wust, J., N.M. Sullivan, V. Hardegger, and T.D. Wilkins.** 1982. Investigation of an outbreak of antibiotic-associated colitis by various typing methods. J. Clin. Microbiol. 16:1096-1101.

43. **Yamakawa, K., S. Nakamura, and S. Nishida.** 1985. Separation of two cytotoxins of Closrtidium sordellii strain. Microbiol. Immunol. 29:553-557.

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PURIFICATION AND CHARACTERIZATION OF *CLOSTRIDIUM SORDELLII* TOXINS HT AND LT AND COMPARISON TO TOXINS A AND B OF *CLOSTRIDIUM DIFFICILE*

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(ABSTRACT)

Clostridium sordellii cause gas gangrene in man and animals, and more recently it has been implicated as a causal agent of diarrhea and enterotoxemia in domestic animals. This organism was once believed to cause pseudomembranous colitis (PMC) in humans, however, *Clostridium difficile*, not *C. sordellii*, was found to be the causative agent of this disease. It is now known that *C. difficile* produces two toxins, designated A and B, that are implicated in the pathogenesis of the disease. *C. sordellii* produces two toxins, designated HT (Hemorrhagic Toxin) and LT (Lethal Toxin), that are similar to toxins A and B of *C. difficile*. The goal of my research was to purify and characterize the two toxins of *C. sordellii*, and compare their properties to those of *C. difficile*.

Toxin HT was purified was purified from *C. sordellii* (VPI strain 9048) culture filtrate by ultrafiltration through an XM-100 membrane filter and immunoaffinity chromatography

using a monoclonal antibody to toxin A of C. difficile as the ligand. Toxin LT was purified to 80% homogeneity by ultrafiltration on an XM-100 membrane filter and ion-exchange chromatography. Toxin HT migrated as a major band with molecular weight of 525,000 and a minor band at 450,000 on non-denaturing PAGE. By SDS-PAGE the molecular weight was estimated at 300,000. Isoelectric focusing indicated a pI of 6.1. Like toxin A, toxin HT was cytotoxic to cultured cells, lethal for mice, and elicited an accumulation of hemorrhagic fluid in rabbit ileal loops. Toxin LT exhibited properties similar to toxin B, although LT was about a 1000-fold less cytotoxic than toxin B. By SDS-PAGE the molecular weight was estimated at 260,000. Immunodiffusion analysis revealed a reaction of partial identity between these toxins and their amino-terminal sequences were very similar.

Toxins HT and LT of C. sordellii have retained remarkable immunological similarities as well as physicochemical and biological properties with toxins A and B of C. difficile; however the toxins are not identical.