Immunogenic studies of Clostridium sordellii
by Miles Huxtable Bairey

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No animal protection to C. sordellii toxin or spores was observed with any of the formalinized products. Protection of mice to low levels of toxin challenge was noted with sublethal toxin injections. Indications of dissociation of the antigen-antibody complex produced by sublethal toxin vaccinations were observed 24 to 48 hours post challenge.

It was concluded from this study that the toxin produced by C. sordellii was the immunizing antigen but when inactivated with formalin, the toxin molecule no longer produced an immunogenic response. It was also concluded that antibody production stimulated by C. sordellii, toxin was of low avidity.

IMMUNOGENIC STUDIES OF CLOSTRIDIUM SORDELLII

by

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ABSTRACT

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INTRODUCTION

Clostridium sordellii was first isolated from a fatal wound infection in man (Sordelli, 1927). It was later isolated in conjunction with other clostridial infections of cattle (Hall, 1927) and Vawter isolated C. sordellii from dead sheep in 1942. Sordelli (1922) named the organism Bacillus oedematis sporogenes, and a similar organism was isolated and named Clostridium oedematoides by Meleney, Humphreys and Carp (1927). The isolated bacteria of Sordelli (1922) and Meleney et al (1927) were found to be identical (Humphreys and Meleney, 1928; Hall, Rymer and Jungherr, 1929) and the name Bacillus sordellii was proposed (Hall and Scott, 1927) to conform with the nomenclature used at that time.

Most work done on C. sordellii relates to morphologic and physiologic considerations in relation to other closely related bacteria, and little has been done on problems relating to antigenicity, immunity, characteristics of toxins, and mode of transmission. Although there are products on the market containing C. sordellii, there are no published data about their efficacy for producing immunity.

This investigation was designed to study the immunizing antigens of C. sordellii and to estimate the possible protection that can be expected from these antigens. Included in this work are the immunizing effects of toxoids, spore bacterins, vegetative cell bacterins, whole culture bacterins and sublethal doses of toxin.

In preparing the present manuscript several studies about taxonomic differentiation and identifications of C. sordellii and C. bifermentans were cited, but this thesis is not a taxonomic study.

REVIEW OF LITERATURE

In 1922, a French surgeon, A. Sordelli, observed an acute fatal edematous wound infection in a human postoperative patient in South America. From this wound he isolated a gram positive, spore producing anaerobic organism. He found that filtrates of supernatant culture fluids were highly toxic to guinea pigs and rabbits and that fluids could not be neutralized by antisera prepared against organisms known at that time to produce gas gangrene. He gave this isolated organism the name _Bacillus oedematis sporogenes_.

Meleney, Humphreys, and Carp (1927) described a similar organism isolated from a postoperative wound infection and from catgut suture material. They produced an antitoxin in rabbits by injecting sublethal doses of 0.05 ml of toxic culture filtrate. This antitoxin would protect white mice against a 0.45 ml subcutaneous (S.C.) injection of toxin produced by this organism. Since they did not recognize that their isolate was identical to the organism isolated by Sordelli, they called their isolate _Clostridium oedematoides_.

During this same period of time, Hall and Scott (1927) studied Sordelli's isolate, performed cultural and pathological studies, renamed the isolate _Bacillus sordelli_, concluded that the lethal factor was a potent exotoxin and confirmed that Sordelli isolated a new species.

The identity of these 2 isolates then became the subject for additional studies. Humphreys and Meleney (1928) reported that

Clostridium oedematoides and B. sordellii were one and the same species

as determined by cross neutralization tests in mice. Their neutralization

was done using rabbit antiserum for each culture. Hall, Rymer and Jungherr

(1929) confirmed the results of Humphreys and Meleney, but used antiserum

prepared by injecting both whole culture material and a sterile filtered

toxin S.C. into rabbits. This was the first description of the anti-

genicity of the toxin. At this time, Hall (1929) was working with some

cultures that he had received from Vawter in Nevada. Vawter had collected

these isolates in 1919 and 1921 from cattle that demonstrated the signs

of bacillary hemoglobinuria. Hall concluded that these isolates were

serologically different from Clostridium hemolyticum but were identical

to Sordelli's isolate from South America and Humphreys, Meleney, and

Carp's isolate from New York.

Subsequently little work was done with this organism until Clark and

Hall (1937) in a comparative study of Bacillus bifermentans and Bacillus

centrosporogenes, the latter organism being described by Hall in 1922,

included B. sordellii in some additional comparison work of closely re-

lated proteolytic anaerobes. They concluded after extensive morphological,

cultural, physiological and serological work, that they could not find

sufficient evidence to separate these 2 species. This was in agreement

with the work of McCoy and McClung (1936) who reported that the H and O

factors of the antigenic complex of 2 strains of Clostridium bifermentans

and one strain of Clostridium centrosporogenes were identical by reciprocal

agglutination absorption experiments. The latter workers concluded that

the 2 species could be combined into a single species, C. bifermentans.
While Clark and Hall (1937) described B. bifermentans as a distinctly
different species from Bacillus sporogenes, Bacillus tyrosinogenes,
Bacillus subterminalis, and Bacillus flabelliferum, they could find no
distinct cultural or morphological difference between B. bifermentans,
and B. sordellii. But because B. sordellii was pathogenic and B.
bifermentans was not, they did not feel that they should be consol-
idated. However, they did observe an antigenic relationship between
these 2 organisms in a weak cross-agglutination reaction. Serum from
some rabbits that had been immunized against B. bifermentans produced
a passive immunity in guinea pigs challenged against either whole
cultures or toxic filtrates of B. sordellii. To confirm their findings
they actively immunized rabbits against B. bifermentans, B. sporogenes
and B. subterminalis and challenged them with B. sordellii culture. They
observed that animals vaccinated against B. bifermentans would withstand
5 to 10 challenge doses more than those animals vaccinated with B.
sporogenes, B. subterminalis or those that were retained as unvaccinated
controls.

While standardizing Clostridium sordellii antitoxin at the National
Institutes of Health in 1938, Stewart observed a relationship between C.
sordellii and C. bifermentans. She prepared a dried toxin and a dried
antitoxin of C. sordellii, and a serum free of antitoxin of C. bifermentans.
She observed that antisera produced against C. bifermentans would protect
the mice against intravenous (I.V.) challenge with 2 to 5 minimal lethal

doses (M.L.D.) toxin. In further studies Stewart learned that the 2 strains possessed identical morphology, colony formation and biochemical reactions, as well as cross-agglutination and cross-precipitation reactions. She concluded from her studies that the 2 species were identical and should be combined under the name of C. bifermentans.

Lillie (1938) reported on the gross and histological pathology produced by artificially infecting mice and other laboratory animals and comparing them to observations in natural infections in man.

Prevot and Cordier (1941) reported that the toxin of C. sordellii was the third most potent toxin produced by a member of the genus Clostridium. They observed that Clostridium botulinum was 20 times more lethal and Clostridium tetani was 4 times more lethal than C. sordellii. Included in this work was a study on antigens shared by C. sordellii and C. bifermentans. They concluded that there were enough differences to separate the 2 organisms by species.

Field isolations of 6 cultures of C. sordellii were reported by Vawter (1942) in Nevada. Of these six, 4 were isolated from cattle along with other Clostridia, one was from a post-vaccinated calf and one was from a sheep; the latter was reported as being non-pathogenic. After studying the cultural, biochemical and serological properties of these isolated organisms, Vawter (1942) concluded that C. sordellii produced a potent moderately stable, filterable toxin in culture fluids. He also reported that the toxin could be inactivated in 20 min at 70 C and would become "anatoxic" when exposed to 0.25% formalin for 2 days at 37.5 C.

He also stated that: "the formalinized cultures were antigenic". He found that filtrates of culture or culture fluids were toxic to horses and that antitoxin could be produced in either rabbits or horses by inject- ing formalinized cultures. In the cross-agglutination work that was done with these strains, it was seen that C. sordellii was antigenically "monovalent". Cross-agglutination occurred not only with these animal strains but also with the human strains that had been isolated in South America. It was also observed that sporulating forms could withstand heating at 90 C and freezing at -70 C as well as dehydration for sever- al years.

A report similar to Vawter's (1942) was made by Pesce-De-Fagonde and Marti-Vidal in 1967 when they isolated C. sordellii from cattle in Argentina that died of icterohemoglobinuria. This bacterium was isolated along with C. hemolyticum. Fasciola hepatica infestation of the liver was also observed.

Other isolations of C. sordellii were reported by Vawter and Smith (1963) in cattle of California feedlots. They reported that these in- fections appeared to be predisposed to by stress factors such as change in temperature, dust and upper respiratory infection.

The work of Vawter (1942) was supported, as was the work of Stewart (1938), by Michael and McClung (1947) when the latter did extensive studies on the flagellar and somatic agglutination of a number of strains of C. bifermentans and of C. sordellii isolated by Hall (1929), Sordelli (1922)

and Meleney et al (1927). They reported that all strains of both species were serologically identical.

Miles and Miles (1947) confirmed the reports of Clark and Hall (1937) and of Stewart (1938) that C. sordellii, C. centrosporogenes, and C. bifermentans were identical and that C. sordellii was but a toxigenic variety. However, after preparing an anti-lecithinase to C. bifermentans they found that this serum would neutralize neither the lecithinase nor the lethal toxin of C. sordellii.

A method for extraction of the C. sordellii toxin from cells was described by Saissac, Renee, and Raynaud (1951). Using this method they reported that the amount of toxin that can be extracted from the cell is of the same magnitude as that found in the broth when grown in a fluid medium. They concluded that this could not be attributed to traces of toxic broth that might be retained by the cells, and that the toxin must be located either inside the cell or on its surface.

Tardieux and Nisman (1952) studied the immunochemical relationship of these 2 organisms. They extracted the polysaccharide fractions from the cells and reacted them with specific antiserum. They found that when subjected to either the "agglutination test" or the precipitation test the polysaccharide fractions would react to high dilutions with homologous antisera. When "cross-agglutination tests" between the 2 species were attempted, there appeared to be no cross-reaction. When gel-diffusion precipitation tests were done against both homologous and heterologous serums, it was found that C. sordellii antiserum would

precipitate the polysaccharide fraction from the cells of both C. sordellii and C. bifermentans whereas C. bifermentans antiserum would precipitate only the homologous extract. They concluded that while there is an immunological relationship between the 2 organisms, they are nevertheless antigenically distinct.

Huet and Aladone first used the urease test in 1952 by testing 200 anaerobic cultures, these represented 176 species of 26 genera. They reported that only 2 species gave an immediate reaction, C. sordellii and Neisseria vulvovaginitis. This produced a differentiating tool which could be readily utilized by workers attempting to classify C. sordellii and C. bifermentans.

Tataki and Huet (1953) used all of the cultures of C. sordellii and C. bifermentans that were in the collection of the Anaerobic Service of the Pasteur Institute in urease studies, and reported that all their cultures of C. sordellii were urease positive. On the other hand, some of the cultures that had been classified as B. bifermentans, because of non-pathogenicity, were also urease positive. Upon submitting these cultures to agglutination reactions they observed that anti-C. sordellii serum would agglutinate not only suspensions of C. sordellii bacteria but also the urease positive strains of C. bifermentans. They also observed that this serum would agglutinate only weakly and intermittently the urease negative strains. However, they also observed that the anti-C. bifermentans serum reacted about the same with all strains indicating that while there were C. sordellii specific antigens, there was also some antigenic similarity.

These authors postulated that since the species had previously been
differentiated by toxigenicity only, some reports of cross-serological
reactions could have been due to the use of non-pathogenic strains of
C. sordellii.

Smith (1955) in a review of the species considered that the serolog-
ical similarities between the 2 organisms were such that C. sordellii
should be considered as a pathogenic variety of C. bifermentans.

Brooks and Sterne (1956) reported isolating an organism that re-
sembled C. sordellii culturally and biochemically from the intestinal
contents of a calf dead from enterotoxemia. When filtered intestinal
contents were injected into guinea pigs, the gross pathology was also
similar to that found with C. sordellii. The toxin isolated could be
neutralized with both the American and the World Health Organization
standard C. sordellii antitoxin. This was the first reported isolation
of this organism in England or the continent of Europe. Whereas the
filtered toxin from the intestinal contents of the dead calf was highly
toxic for guinea pigs, filtrates of the cultures grown from the isolation
when reinjected into calves produced no pathogenicity.

This same year, Meisel (1956) worked with the toxins of C. sordellii
and observed that the amount of toxin produced by a culture could be
increased by incorporating glucose into the medium. These studies showed
a cross-reaction with C. welchii (C. perfringens) antitoxin and it was
observed that this antitoxin neutralized the lecithinase of C. sordellii.

It was from this work that Meisel reported that in addition to lecithinase and hemolysin, C. sordellii contained a necrotic and lethal toxin.

Meisel and Rymkiewicz (1958) hypothesized that endospores of proteolytic clostridia contained not only the spore antigen, but also the H and O antigens of the vegetative cells. To prove this theory, they treated endospores of C. tetani and C. sporogenes with lysozyme, desoxyribonuclease and trypsin. These enzyme treated spores were then autoclaved for 40 min at 120 C, injected into rabbits and an antiserum produced. The antiserum agglutinated not only the spore antigen, but also H and O vegetative antigens. By comparison, antiserum to either H or O vegetative antigens would not agglutinate this suspension. They concluded that the endospore was not an H or O antigen but could stimulate the production of specific antibodies in the rabbit.

Brooks and Epps (1958) collected 62 different strains from various sources. Using the differentiating criteria of pathogenicity and urease production, they found that they had 24 non-pathogenic, urease negative strains which they called C. bifermentans; 13 strains that were pathogenic and urease positive, classified as C. sordellii (P); and 25 strains that were non-pathogenic but were urease positive and considered to be C. sordellii (N-P). When these cultures were inoculated into glucose, fructose, maltose, mannose, glycerol, sorbitol and salicin these workers observed that the C. bifermentans fermented all of them with the exception of one strain not fermenting sorbitol and one not fermenting salicin.

Both $\underline{C}$. sordellii (P) and $\underline{C}$. sordellii (N-P) fermented glucose, fructose,
maltose and glycerol but none of the other sugars. This resulted in a
differentiation of the 2 species by sugar fermentations. These strains
were also submitted to chromatographic examination and it was observed
that $\underline{C}$. sordellii produced large amounts of histidine while $\underline{C}$.
bifermentans strains produced none. It was their conclusion that
the 2 species could be separated on toxigenicity, urease production and
ability to ferment mannose, salicin, and sorbitol.

In 1960, Norris and Wolf studied several strains and species of the
genus Bacillus and observed that antiserum produced against unheated spores
would agglutinate unheated spore antigen, autoclaved spore antigen, and
vegetative H antigen but not the vegetative O antigen. Antiserum pro-
duced against autoclaved spores would agglutinate autoclaved and unheated
spore antigen but neither vegetative H nor O antigen. However, antiserum
to vegetative H antigen would agglutinate unheated spore antigen but not
autoclaved spore antigen. In no instance would antiserum produced against
vegetative O antigen agglutinate a spore antigen. Injection of H antigen
into rabbits would stimulate the production of both H and O antibodies.
It was interesting to note that these workers reported that antiserum
produced against spores treated with formalin would not agglutinate
either H or O antigen.

Crane and Poulos (1962) reported that $\underline{C}$. sordellii was isolated from
feedlot cattle in pure culture in California. These authors postulated

that this organism may be a normal inhabitant of the ruminant diges-

tive tract.

Two isolations of C. sordellii from sheep were reported by Smith,

Safford, and Hawkins (1962). The first isolation was made from a ram

that died 10 days after being sheared and the second was from 2 rams

which had edematous heads and necks. It appeared to these authors that

the pathogenesis of this organism may have been due to a wound infection

in the first instance and to tissue traumatization as a result of

fighting in the latter case. Whereas the isolation of C. sordellii

from affected tissue was accomplished easily, it could not be isolated

from the soil. Soil sample isolations produced only non-pathogenic,

urease negative, C. bifermentans.

Walker (1963) studied spore suspensions of C. sordellii and C.

bifermentans that were produced by growing the organism for 3 to 4

days at 37 C, washing the culture in distilled water and centrifuging

at 3,000 r.p.m. 6 times, resuspending in water and shaking vigorously,

recentrifuging, removing the supernatant fluid and diluting to a Brown

opacity standard number 8. After microscopic examination for purity,

the spores were autoclaved for 20 min at 121 C to prevent germination

and production of H and O antigens. Vegetative cell antigens were pre-

pared by harvesting 12 to 16 hr cultures, centrifuging, washing and

standardizing them in the same method as the spores were handled. The

H antigen was prepared by submitting this vegetative cell preparation to

incubation in 0.08% formaldehyde for 2 hrs at 37 C. The O antigen was

prepared by steaming the suspension for one hr. Walker observed that C. sordellii and C. bifermentans could be differentiated on the basis of their spore agglutinins but not on their precipitins.

Six distinct antigens of C. bifermentans were determined by Ellner and Green (1963); 5 of these were common to all 4 of the strains used. Five antigens of non-pathogenic C. sordellii and 7 antigens of pathogenic C. sordellii were also found. Five of the latter were common to all of the 9 strains used. Agar-gel diffusion and antigen-antibody absorptions were the methods that were used to distinguish these antigens. These experiments clearly distinguished between pathogenic and non-pathogenic strains. These workers also found that there were 4 antigens common to both C. sordellii and C. bifermentans with the former having, in addition, 3 species specific antigens and the latter 2.

Nishida, Tamai and Yamagishi (1964) studied species differentiation between C. sordellii and C. bifermentans in a somewhat different manner. From previous work on C. perfringens, (Yamagishi, Ishida and Nishida, 1964), they postulated that the ability to sporulate was closely associated with toxigenesis. They observed that strains of the non-pathogenic C. bifermentans sporulated to a greater degree than did the pathogenic strains of C. sordellii. They identified their strains by toxigenicity and urease production and submitted them to heat treatments. They reported that 7 heat-resistant strains of pathogenic C. sordellii became non-toxigenic when exposed to 90 C or 100 C for 10 min. Some of these heat-resistant strains were urease positive and resembled

non-pathogenic C. sordellii, while others became urease negative and resembled C. bifermentans. The sporulation of C. bifermentans was high, that of pathogenic C. sordellii was low and that of non-pathogenic C. sordellii was intermediate between the 2. Tamai and Nishida (1964) continued studies on effects of heat treatment. Two new strains of pathogenic C. sordellii were studied and in one strain, toxigenicity was lost after heat treating at 80 C for 30 min. In the original culture, urease activity was lost at this time also but in a substrain the urease activity was not lost until it was heated at 90 C for 30 min. In another strain, a C. bifermentans-like substrain could not be obtained by heat selection. Huang, Tamai and Nishida (1965) continued this work and added the use of agglutination tests to support their evidence. They reported that the heat-resistant substrains of 3 pathogenic C. sordellii strains not only lost their toxigenic properties but also lost their species specific antigens and acquired the antigenic properties of C. bifermentans. They used cross-agglutination tests and agglutination-absorption tests. These authors concluded that their work proved the close relationship of the 2 species. That, they had converted a pathogenic C. sordellii organism into a C. bifermentans further proved the 2 species were actually one and the same.

Prevot (1966) in the first American edition of the Manual for the Classification and Determination of the Anaerobic Bacteria, classified C. sordellii as a variety under the subgenus Clostridium sporogenes

and differentiated it from C. bifermentans on pathogenicity, urease
production, sugar fermentation and growth products.

The pathology, along with the pathogenesis of C. sordellii was
again studied by Wren in 1968 in guinea pigs and cattle. He found that
any parenteral route of administration would produce death in either
species but oral administration by several methods would not. He did
observe that guinea pigs allowed to drink contaminated water would
shed viable organisms for as long as 20 days, indicating the possibility
of growth of the organism within the digestive tract without intoxicating
the animal. Wren's studies bring up the possibility of carrier animals.

MATERIALS AND METHODS

Experimental Animals.

White Swiss mice of both sexes weighing 18 to 22 g used in the various studies were obtained from the Veterinary Research Laboratory animal colony at Montana State University and from a commercial breeder (Carworth Inc. Portage, Michigan). Guinea pigs of both sexes weighing approximately 300 g were used for pathogenic determinations of C. sordellii strains. The guinea pigs were obtained from the animal colony at Montana State University.

Selection of Strains.

Several strains of C. sordellii were acquired from the Veterinary Biologics Division of the Agricultural Research Service at the National Animal Disease Laboratory in Ames, Iowa and from Dr. Louis DS. Smith at Montana State University, Bozeman, Montana. These strains were transferred from stock cultures to an anaerobic liver broth medium and incubated anaerobically at 37 C. After growth for 19 hrs stained smears of the organisms were observed microscopically for a preliminary determination of purity. Actively growing cultures were streaked on freshly poured bovine blood agar plates and egg yolk agar plates to observe colonial morphology and to establish hemolytic and lecithinase activity. These plates were incubated anaerobially in Brewer jars at 37 C for 4 days as described by Smith (1955).

In order to determine carbohydrate fermentation, various sugars sterilized by filtration were added to tubes of freshly prepared

thioglycollate medium to give a final concentration of 0.5%. These tubes were then boiled for a few minutes to drive off oxygen and to mix the added carbohydrates with the thioglycollate broth. The cooled tubes were inoculated with approximately 0.5 ml of broth culture, using sterile Pasteur pipettes fitted with rubber bulbs. The inoculated carbohydrate tubes were incubated aerobically at 37 C for 3 days and then observed for fermentation by the addition of a loopful of culture to a pH indicator (1% Brom Thymal Blue) placed on a spot plate. These cultures were re-incubated for an additional 4 days, and at that time the indicator solution was added to the culture for a final assay of carbohydrate fermentation.

The strains of C. sordellii were examined for urease production by inoculating a medium composed of a 31% Urea Broth (Difco, 0272-02) solution mixed 1 to 8 with thioglycollate broth (BBL, 411 665). A heavy inoculum acquired by scraping bacterial growth from a blood agar plate with a wire loop was used to inoculate this medium. These tubes were incubated aerobically at 37 C for 4 hrs.

Cultures were selected for toxigenicity by growing in anaerobic liver broth for 21 hrs, centrifuging to remove the meat particles, and treating the supernatant fluid with 100 mg of aqueous penicillin per ml. A volume of 0.2 ml fluid was injected intraperitoneally (I.P.) into white Swiss mice. Whole culture material was mixed one to one with sterilized 5%

calcium chloride solution and 2.5 ml was injected intramuscularly (I.M.)
into guinea pigs to determine the pathogenicity of the strains.

Four strains that produced hemolysis on blood agar plates, fermented
glucose, fructose, and maltose but not mannose, salicin or sorbitol were
selected for further studies for toxin production. All were considered
toxigenic because they killed mice within 24 hrs when injected I.P. with
penicillin treated supernatant fluid of broth cultures. These 4 strains
were pathogenic for guinea pigs, produced urease and with the exception
of one strain, produced lecithinase. Throughout the rest of this
manuscript these strains will be known as A-125, 831, 1732 and 3903.
The latter strain did not produce lecithinase.

Culture Media Used.

Growth culture medium. The medium selected for routine culture
growth and used as a base for other media was a liver infusion broth
to which was added 1% trypticase (BBL), 0.5% yeast extract (Difco),
0.5% dextrose and 0.01% magnesium sulfate (Lozano, 1965). Into 16 ml
tubes were placed 8 ml of this medium and a small amount of dried, ground
beef muscle and a pea-sized ball of steel wool. This medium was adjusted
to a pH of 7.4, and will hereafter be referred to as standard growth medium.

Method for determining effect of length of incubation on toxin yield.
The medium used was the standard growth medium. On the first trial, the
4 strains of C. sordellii were incubated in 4 separate Brewer jars
anaerobically at 37 C, 2 cultures of each strain per jar. The cultures

were harvested at 6-hr intervals starting at 6 hrs after inoculation. The toxic fluids were prepared and injected into mice. On the second trial, the 6-hr growth period was eliminated and 30 and 36 hr periods were added.

Method of estimating effect of growth temperature on toxin production. The procedure was to inoculate cultures of each of 2 selected strains into standard growth medium, and place them under anaerobic conditions. The cultures were incubated in individual incubators (Napco 1000 series, Model 320) at either of the following temperatures 30 C, 33 C, 37 C and 42 C. The cultures were removed at 18 hrs and the toxic fluids prepared and injected into mice. A second trial was conducted, eliminating the 2 lowest temperatures and adding one higher temperature, 45 C.

Method for studying effect of peptone concentration on toxin yield. To vary peptone concentration of the medium, standard growth medium had to be altered to eliminate the other components that would contribute peptones. The peptone variation broth used was made with 0.5% dextrose, 0.5% sodium phosphate and either 2%, 3%, 4% or 5% Bacto-tryptose (Difco) in distilled water. This preparation was autoclaved for 15 min at 121 C, and then the pH adjusted to 7.4. Tubes of each of these peptone concentrations were inoculated with strain 1732 and incubated at 42 C and harvested at 18 hrs. Tubes were also inoculated with strain 3903 at each of these peptone concentrations, incubated anaerobically at 37 C and harvested at 18 hrs.

Method for studying effect of dextrose concentration on toxin yield.
The medium used for this determination was the same as the peptone
variation procedure except that the peptone concentration was maintained
at 4% and the dextrose concentration was varied. The dextrose concentra-
tions used were 0.5%, 1.0%, 1.5%, 2.0% and 2.5%. Both strains were in-
cubated anaerobically for 18 hrs before the toxic fluids were harvested
and injected into mice. Strain 1732 was incubated at 42 C, whereas
strain 3903 was incubated at 37 C.

Toxin production medium. Toxin production flasks were prepared by
placing 16 g tryptose (4% peptone), 2 g dextrose (0.5%) and 2 g sodium
phosphate (0.5%) into 5 liter Erlenmeyer flasks. Four liters of distilled
water were added and mixed until all of the dry ingredients were in
solution. The preparation was autoclaved for 20 min at 121 C. The pH
was adjusted to 7.4 by the addition of 0.1 N sodium hydroxide.

Spore production medium. It had previously been described in the
literature that when C. sordellii was grown on a solid medium for ex-
tended periods of time the colonies consisted predominantly of spores
with few vegetative cells (Smith, 1955). By microscopic examination of
Gram's stained smears of colonies, it was determined that after 4 days
growth on a 5% bovine blood agar plate there was a predominance of free
spores with only occasional vegetative cells.

Method of Preparation and Storage of Toxic Materials.

Crude toxin. This toxin was prepared as follows: decanting toxic fluid from toxin production cultures, centrifuging (International Centrifuge Size 1, Type SB) at 545 X g, and filtering the supernatant fluid through a 0.45 μ Millipore filter. Crude toxin preparations were discarded if not used immediately.

Semi-purified toxin. Five seed cultures containing 50 ml of standard growth medium were inoculated with strain 1732 and incubated anaerobically in Brewer jars for 20 hrs at 37 C.

Five production flasks were inoculated by transferring the contents of one seed culture tube into each production flask. The transfer was made after flaming the mouth of both the tube and the flask and passing the contents through sterile cheesecloth to remove the meat particles. The production flasks were then incubated at 42 C for 18 hrs.

The 20 liters of 18-hr growth was harvested by centrifuging (Sorvall SS3 Automatic Superspeed Centrifuge) at 1,000 X g for 20 min and decanting and combining the supernatant fluids in 2 sterile pans. Fifty per cent w/v of ammonium sulfate was mixed into the supernatant fluids and stirring was continued until no more flotsam was formed. This procedure was carried out at 4 C. The toxin was recovered by skimming the flotsam that had risen to the top with a stainless steel screen and placed in 250 ml round bottom flasks. The contents in the round bottom flasks were shell frozen and lyophilized (Virtis Research Equipment).

The dried toxin was removed from the round bottom flasks, pooled into one container and placed in a desiccator jar containing sodium carbonate. Dried toxin inoculated into standard growth medium and incubated at 37 C anaerobically produced no growth and was considered to be free of viable organisms. This was considered to be a semi-purified preparation of C. sordellii toxin.

Spore suspension. Twenty-seven 5% bovine blood agar plates were heavily inoculated and incubated at 37 C anaerobically in Brewer jars for 4 days. Harvest of the spores was done by the method of Long and Williams (1958). The method consisted of scraping the colonies from the agar with a wire loop and washing the agar surface with 0.1 N sodium phosphate buffer. This suspension was centrifuged (Sorvall SS3) at 1885 X g for 30 min. The supernatant fluid containing the vegetative cells was decanted and the precipitated button was rewashed with 0.1 N sodium phosphate buffer and recentrifuged. This process was repeated 4 times. The final suspension was aerated by shaking and refrigerating overnight at 4 C to increase lysis of any vegetative cells which might be remaining. The following morning the spores were washed and centrifuged as before 5 more times and suspended in 60 ml of buffer solution. The spore suspension was examined several times microscopically and no vegetative cells were observed.

The suspension was pipetted into twelve 10 ml freeze-drying ampules, 5 ml to each ampule and lyophilized. The ampules were sealed by heat under vacuum and stored at -20 C.

Methods of Determining Toxigenicity and Pathogenicity.

Toxin preparation. Mouse lethality was used as the criterion for toxicity of C. sordellii toxin preparations. White Swiss mice weighing 18 to 22 g were given I.P. injections of toxins. All tests were held for 24 hrs and only mortality rates were used to determine effects.

Spore suspensions. Mouse lethality was used as the criterion for pathogenicity of spore preparations. Spore suspensions were injected I.M. into the large muscles of the rear legs along with calcium chloride. Spore titrations were terminated after 48 hrs and only live and dead animals were used in the determinations.

Dilution methods. Toxin dilutions were made in 1% Bacto-peptone (Difco 0118-01) water whereas spores were suspended in sterile distilled saline. The lethality titration dilutions were made in 2-fold, 10-fold or 0.0025 mg increments of the toxic preparations. Lethal doses were calculated by the method of Reed and Muench (1938). All dilutions were made by changing pipettes with each dilution and mixing the preparation by carefully drawing the solution into a pipette and exuding it 10 times for each tube.

Method of Preparing Immunizing Materials.

Toxoids. Toxoids were prepared by diluting dried toxin in 1% peptone water to give 3 preparations of 1000 M.L.D.s per 0.5 ml dose, 100 M.L.D.s per 0.5 ml dose and 10 M.L.D.s per 0.5 ml dose respectively. To each of these preparations a sufficient amount of 36.9% formalin was added and thoroughly mixed by horizontal motion to give a final dilution of 0.7% formalin. The formalinized toxin preparations were incubated at 37 C for 4 days to insure complete inactivation of the toxin. The pH of these toxoids was adjusted to 7.4. Sterility tests were performed on toxoids by heavily inoculating 5 tubes of standard growth medium and incubating 3 tubes anaerobically and 2 tubes aerobically for 10 days at 37 C. Daily observations for evidence of lack of growth and terminal microscopic examinations of stained smears were used for these sterility tests. Safety tests to determine product inactivation were performed by 0.2 ml I.P. injections in white Swiss mice and by 2.5 ml S.C. injections in guinea pigs. Fluids used for guinea pig safety inoculations were mixed with 5% calcium chloride. Satisfactory toxoids were stored at 4 C.

Spore Bacterins. Preparation of spore bacterins was similar to that of the toxoids. The calculated amount of spores that would give 3 preparations of 1000 M.L.D.s per 0.5 ml dose, 100 M.L.D.s per 0.5 ml dose and 10 M.L.D.s per 0.5 ml dose respectively was suspended in sterile distilled saline. Formalin was added to give a final concentration

of 0.7% formalin. The formalinized preparation was incubated for 4 days at 37 C, the pH was adjusted to 7.4 and sterility tests were performed by the same procedure used for the toxoids. Safety tests were carried out by the same method as with the toxoids to insure that the spores were inactivated. Satisfactory bacterins were stored at 4 C.

Vegetative cell bacterins. To produce a washed cell bacterin it was determined at what phase of growth spores began to form in order to harvest the vegetative cells prior to sporulation. It was necessary to determine at what phase of growth vegetative cells became the most virulent for mice.

Twelve test tubes of standard growth medium were inoculated with strain 1732 and incubated anaerobically at 37 C and 2 tubes were removed from incubation at 4-hr intervals for examination.

Washed cells were processed by pouring cultures through sterile cheesecloth. After centrifuging (Sorvall SS3) at 130 X g for 10 min, removing the supernatant fluid carefully and resuspending the pelleted cells in an equal volume of sterile saline, the resultant suspension was recentrifuged. This procedure was carried out 4 times. The final cell pellet was resuspended in enough sterile saline to produce a Macfarlane nepholometer reading of 8 which was the same as the broth culture before washing the cells. A Bausch and Lomb Spectronic 20 spectrophotometer adjusted to 740 mμ was used to evaluate nepholometer readings.

This suspension of washed cells with a Macfarlane nepholometer reading of 8, was diluted to 10^{-3} and 10^{-8}. The washed cell bacterins were prepared with cultures from ten 50 ml tubes of standard growth medium that were inoculated with strain 1732 and incubated anaerobically at 37 C for 10 hrs. The pH was adjusted to 7.4. These 2 preparations were inactivated with formalin and tested for sterility and safety in the same manner as the toxoids and the spore bacterins. Those found to be satisfactory were stored at 4 C.

Whole culture bacterins. The whole culture bacterin was prepared by inoculating ten 50 ml tubes of standard growth medium and allowing them to incubate for 10 hrs anaerobically at 37 C. The cultures were poured through sterile cheesecloth to remove meat particles. Dilutions were made to 10^{-3} and 10^{-8} and treated with formalin to inactivate them. The pH was adjusted to 7.4 and sterility and safety tests were performed as described previously. Satisfactory bacterins were stored at 4 C.

Method for Heat Inactivation.

The production of heat resistant substrains of C. sordellii strain 1732 was attempted by the method of Nishida, Tamai and Yamagishi (1964). This consisted of inoculating test tubes of standard growth medium with strain 1732 and allowing them to grow anaerobically at 37 C for 20 hrs. The tubes were placed in water baths adjusted to 80 C, 90 C or 100 C. Cultures removed from the water baths were immediately transferred to

.freshly boiled standard growth medium and incubated anaerobically at 37 C for 18 hrs. These cultures were injected into mice to determine if they were pathogenic and were inoculated into urea test medium to see if there was urease production (Tamai and Nishida, 1964: Huang, Tamai and Nishida, 1965).

Serological Methods.

Precipitation reactions between toxins, toxoids and specific antiserum were developed in a plate gel-diffusion system according to Ouchterlony (Ouchterlony, 1968, Campbell et al, 1964).

EXPERIMENTAL RESULTS

Preparation of Toxoids.

Factors affecting toxin yield. To facilitate the productions of toxin, several variations were made in the procedures used in toxin production. Also selection of a high toxin producing strain of C. sordellii was necessary.

Preliminary trials indicated that strains of C. sordellii, under these conditions would reach their peak of toxin production at 18 hrs and would maintain this level of production for 24 to 36 hrs depending on the strain.

The temperature of incubation was studied with respect to toxin production using only strains 1732 and 3903. These particular strains were chosen because they produced a potent toxin with regularity.

After 18 hrs growth it was learned that 42 C was the optimal toxin production temperature under these conditions for strain 1732, and 37 C for strain 3903.

The third factor considered was the effect of concentration of peptone on toxin production. The results of the peptone variation studies are summarized in Table I. It may be observed that there was little difference in toxin production with strain 1732 when 3% to 5% peptone was used in this medium. Similar observations were made with strain 3903.

Table I. Effects of peptone concentration on toxin yield

% Peptone	Lethality in mice (dead/total injected)					
	Strain 1732			Strain 3903		
	1:100	1:500	1:1000	1:100	1:500	1:1000
2%	2/2	0/2	0/2	2/2	2/2	1/2
3%	2/2	1/2	2/2	2/2	0/2	0/2
4%	2/2	2/2	2/2	2/2	2/2	0/2
5%	2/2	1/2	2/2	2/2	2/2	0/2

In studies of 5 different concentrations of dextrose, the results of testing at 0.5% to 2.5% in 0.5% increments are recorded in Table II. Since it was desirable to work with only one strain, C. sordellii 1732 was selected because it produced the most toxin under varied conditions. The optimal medium for toxin production with strain 1732 was determined to be: 0.5% dextrose, 4% peptone, 0.5% sodium phosphate buffer in distilled water.

Twenty liters of satisfactory toxin was produced in production medium and the toxin was extracted with ammonium sulfate, lyophilized and stored.

Toxin titration. Before a toxoid could be made, it was necessary to determine the toxicity of the lyophilized toxin preparation. Serial

Table II.   Effects of dextrose concentration on toxin yield

% Dextrose	Lethality in mice (dead/total injected)									
	Strain 1732					Strain 3903				
	1:100	1:400	1:800	1:1000	1:5000	1:100	1:400	1:800	1:1000	1:5000
0.5%	2/2	2/2	2/2	2/2	1/2	2/2	2/2	2/2	2/2	0/2
1.0%	2/2	2/2	2/2	2/2	0/2	2/2	2/2	0/2	0/2	0/2
1.5%	2/2	2/2	2/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2
2.0%	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
2.5%	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

2-fold dilutions were made of this solution to give 7 dilutions, containing 0.1 mg to 1.56 X 10^{-3} mg, which were injected I.P. into 2 mice each as a screening test. It was observed that all injected mice died indicating a higher level of toxicity than had been anticipated. A second titration was conducted with reduced amounts of toxin using 0.01 mg per 0.1 ml dose for the uppermost dilution level. This experiment was repeated using 5 mice in place of 2 mice per dilution. The results of preliminary toxin titrations are summarized in Table III.

Table III. Preliminary toxin titrations

Trial	Mg of toxin							
	0.01	0.0075	0.005	0.0025	0.001	0.00075	0.0005	0.00025
* #2	***1/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2
** #3	ND	ND	5/5	5/5	4/5	2/5	2/5	ND
Total	1/2	2/2	7/7	7/7	5/7	2/7	2/7	0/2

* #2 test is the screening test using 2 mice per dilution.

** #3 test is the screening test using 5 mice per dilution.

*** Dead mice/total mice injected.

ND Denotes that the test was not done at this dilution.

It can be seen from the results that the M.L.D. of this toxin preparation was 0.0025 mg per mouse dose when given I.P.

Inactivation of toxins. To insure that when the toxoids were prepared there would be adequate toxin available, the next higher dilution above the titrated M.L.D. was taken as the "toxoiding" dose. Toxoids were thus prepared using 0.005 mg per dose. Inactivation of toxin was accomplished as previously described.

Preparation of Spore Bacterins.

Spore suspension production. It was determined that the optimal method to produce large numbers of spores was growth for 4 days on solid media.

Spore suspension titrations. Preliminary titrations of lyophilized spores suspended in 2.5% calcium chloride resulted in death of all mice. Several calcium chloride concentrations were injected I.M. into mice in various volumes. No deaths occurred, eliminating the possibility that death was caused by the calcium chloride.

Further studies were then conducted using various concentrations of spores suspended in either of 3 different concentrations of calcium chloride or sterile distilled saline. The effect of volume of the injections was also studied. The results of these experiments are recorded in Table IV and show that the concentration of calcium chloride used with the spore suspension did make a difference in the lethality. With the smaller volume, 1%, there did not appear to be sufficient

Table IV. Effect of dosage and calcium chloride on pathogenicity of spores

Vol. in ml/$CaCl_2$	Spore prepartion in mg											
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
0.1/H_2O	*0/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.1/1.0	1/2	1/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.1/2.5	2/2	2/2	2/2	2/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.1/5.0	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2
0.3/H_2O	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
0.3/1.0	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2
0.3/2.5	2/2	2/2	2/2	2/2	2/2	2/2	1/2	0/2	0/2	0/2	0/2	0/2
0.3/5.0	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	1/2	2/2	0/2	1/2

*Dead mice/total mice injected.

calcium chloride to produce suitable conditions for spores to exert
their lethal effect. At the 0.3 ml volume there was a significant and
uniform relationship between the calcium chloride concentrations and
lethality. In the absence of calcium chloride, germination of spores
may not take place in sufficient amounts to cause death. It was de-
termined that the 2.5% calcium chloride concentration gave a satisfactory
level of anaerobiosis and would be used with spore suspensions hereafter.

In the previous experiment it was observed that there was significant
differences between a 0.1 ml dose and 0.3 ml dose containing the same
amount of spore suspension with the same concentrations of calcium chloride.
Because the injections were made I.M. and the size of the test animal was
small, it was desirable to use a small dose. To further study the effect
of volume, an experiment was done to compare a 0.3 ml dose to a 0.2 ml
dose, both containing the same amount of spore suspension and both
having a final concentration of 2.5% calcium chloride. The results of
this work are given in Table V. It was learned that the 0.2 ml dose
was at least as effective as the 0.3 ml dose. This volume was used for
subsequent spore injections into mice.

Table V. Effect of dosage volume on pathogenicity of spore suspension

Volume injected in ml	Spore suspension in mg				
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
0.2	*5/5	5/5	1/5	1/5	0/5
0.3	5/5	2/5	0/5	0/5	0/5

*Dead mice/total mice injected.

With volume and concentration of the dose and the concentration of calcium chloride established, an experiment to determine a M.L.D. was performed. The results of these tests are summarized in Table VI.

There was considerable variation in results, but it was observed that in 3 of the 5 titrations the M.L.D. was 1.25×10^{-6} mg and this dose was selected for the preparation of spore bacterins.

Preparation of Vegetative Cell Bacterins.

Vegetative cell titrations. Vegetative cells of strain 1732 of C. sordellii were washed and prepared for pathogenicity titrations as described previously. Ten-fold dilutions were made of the final washed cell suspension and 8 dilutions each were injected into 10 white Swiss

Table VI. <u>C. sordellii spore suspension titrations</u>.

Repetition	Spore suspension in mg										
	*100	50	25	12.5	6.25	3.125	1.563	0.78	0.39	0.195	0.098
#1	**5/5	4/5	4/5	4/5	4/5	3/5	1/5	1/5	1/5	1/5	0/5
#2	5/5	5/5	5/5	5/5	3/5	3/5	1/5	1/5	1/5	2/5	1/5
#3	ND	ND	4/5	4/5	3/5	2/5	0/5	0/5	ND	ND	ND
#4	5/5	5/5	5/5	5/5	3/5	3/5	2/5	3/5	ND	ND	ND
#5	5/5	5/5	5/5	5/5	5/5	4/5	4/5	3/5	0/5	0/5	ND
Total	20/20	19/20	23/25	23/25	18/25	15/25	8/25	8/25	2/15	3/15	1/10

* All numbers times 10^{-7}.

** Dead mice/total mice injected.

mice I.M. using a 0.2 ml dose with a final concentration of 2.5%
calcium chloride. The results of these injections are summarized in
Table VII and indicate that the organisms from 10-hr growth were more
pathogenic. Bacterins were made from dilutions of this preparation.

Table VII. Lethality titrations of vegetative cell preparations

Growth in Hours	*Dilutions							
	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
6 hr	**10/10	10/10	10/10	10/10	7/10	2/10	0/10	0/10
10 hr	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10

*
Dilutions were prepared from vegetative cell suspensions that had a
Macfarlane nepholometer reading of 8.

**
Dead mice/total mice injected.

Preparation of Whole Culture Bacterins.

Whole culture production. Several tubes of standard growth medium
were inoculated with strain 1732 of C. sordellii. These tubes were
incubated anaerobically at 37 C, one test tube was removed every 4 hrs,
starting at 6 hrs post-inoculation.

Whole culture titrations. Cultures from the test tubes were diluted
in the same broth medium that the cultures were grown in making 10-fold

dilutions. The dilutions were injected I.M. into 10 white Swiss mice

per dilution with a final concentration of 2.5% calcium chloride.

No end point was reached and the 6, 10 and 14-hr incubation periods

were not significantly different. The 18-hr culture had a definitely

lower end point and was not included in a second trial. The results

of the second trial are summarized in Table VIII and show that the

whole culture was most pathogenic after a 10-hr incubation period.

Bacterins were subsequently prepared from cultures incubated 10 hrs.

Table VIII. Lethality titration of whole culture C. sordellii

Growth in Hours	*Dilutions							
	10^4	10^5	10^6	10^7	10^8	10^9	10^{10}	10^{11}
6 hr	**10/10	10/10	10/10	9/10	5/10	4/10	1/10	0/10
10 hr	10/10	10/10	10/10	10/10	10/10	3/10	3/10	0/10
14 hr	10/10	9/10	10/10	8/10	4/10	2/10	1/10	0/10

* Dilutions were made from strained whole cultures.

** Dead mice/total mice injected.

Comparison Studies of Immunizing Ability of C. sordellii Products.

Several C. sordellii products were produced that were suspected of

having immunizing ability against homologous or heterologous challenge

with C. sordellii toxin or spores. Three of these products were toxoids produced with different concentrations of lyophilized toxin. Three were spore bacterins prepared with different concentrations of spore suspensions. Two were bacterins prepared from 2 different concentrations of washed vegetative cells and 2 were bacterins prepared from 2 different concentrations of whole cultures. Commercially prepared aluminum hydroxide precipitated whole culture bacterins were also used. These products were acquired from the National Animal Disease Laboratory in Ames, Iowa and designated by the code numbers B9425 and D9764.

Toxin challenge determinations. Animals vaccinated with various products were challenged with one M.L.D. of toxin. The M.L.D. was determined in mice given 2-fold dilutions of toxin. The results of these titrations and a comparison between I.P. and I.V. injections are summarized in Table IX.

It was observed that the M.L.D. of the toxin when given I.P. was 5×10^{-3} mg. The M.L.D. by the I.V. route was greater than 5×10^{-3}. The I.P. route was selected as the route of administration of the toxin challenge.

Spore challenge determinations. It was decided to challenge at or near the M.L.D. Calculations were made from titrations recorded in Table VI. The selected challenge dose of the spore suspension was 5×10^{-7} mg.