Anaerobe 2010

The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

SESSION XI: AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

TOLL-LIKE RECEPTORS IN THE INNATE IMMUNE RESPONSE TO *CLOSTRIDIUM SORDELLII*

Aldape, M.J.;*[1] Bryant, A.E.;[1] Katahira, E.J.;[1] Hajjar, A.M.;[2] Finegold, S.M.;[3] Stevens, D.L.[1,2]
[1]Veterans Affairs Medical Center, Boise, ID USA
[2]University of Washington, Seattle, WA USA
[3]Veterans Affairs Medical Center, Los Angeles, CA USA

Clostridium sordellii has recently been associated with rapidly fatal infections following medically-induced abortions and injecting drug use. Patients with *C. sordellii* infection display few signs of inflammation such as fever, or redness and pain at the site of infection. We hypothesized that this could be due to reduced recognition of the organism by Toll-like receptors (TLRs) of the innate immune system. An ELAM-NF-κB luciferase reporter system in TLR-transfected HEK cells was used to measure TLR-dependent recognition of washed, heat-killed *C. sordellii* and other pathogenic clostridial species. Results demonstrated that all clostridia were well recognized by TLR2 alone and that responses were greatest when TLR2 was co-expressed with TLR6. Further, isolated human monocytes produced the pro-inflammatory cytokine TNFα and the immunoregulator IL-10 in response to *C. sordellii*. In addition, *C. sordellii*-stimulated monocytes produced 30% less TNFα following treatment with an anti-TLR2 blocking antibody. These data demonstrate that innate immune recognition of, and response to, cell-associated components of *C. sordellii* and other clostridial pathogens are mediated by TLR2 in combination with TLR6. We conclude that the characteristic absence of inflammatory signs and symptoms in *C. sordellii* infection is not related to inadequate immune detection of the organism, but rather is attributable to a species-specific immune system dysfunction that remains to be elucidated.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

THE IMPORTANCE OF CLASS A SCAVENGER RECEPTORS IN THE PHAGOCYTOSIS OF *CLOSTRIDIUM SORDELLII*

Aronoff, D.M.*
Graduate Program in Immunology, Program in Biomedical Sciences, Division of Infectious Diseases, Department of Internal Medicine, The University of Michigan Health System, Ann Arbor, MI USA

Background: *C. sordellii* is a toxigenic anaerobe and an uncommon cause of highly-lethal female reproductive tract infections, particularly after childbirth, abortion, or cervical procedures. Advances in the treatment and prevention of these infections are limited by gaps in our knowledge of the interactions between *C. sordellii* and the immune system. These studies sought to determine how human uterine macrophages recognize and internalize *C. sordellii*.
Objectives: To define the major phagocytic pathways for unopsonized, vegetative *C. sordellii* by macrophages of the human female reproductive tract.
Methods: Decidual macrophages (DMs) were isolated from adult women undergoing uncomplicated first trimester surgical abortions. Real time PCR was performed to assess the level of expression of class A scavenger receptors by DMs. *C. sordellii* strain ATCC9714 was heat-killed and labeled with a fluorescent dye for studies of phagocytosis. DMs were pretreated with antagonists of CASRs before challenge with unopsonized *C. sordellii*. *In vitro* phagocytosis assays were also conducted with peritoneal macrophages (PMs) from scavenger receptor AI/II (SR-AI/II) knockout (KO) mice and MARCO receptor KO mice. *In vivo* intrauterine infection studies with *C. sordellii* were performed in wild-type (WT) or MARCO KO mice.
Results: Human DMs express the CASRs SR-AI/II, SRCL, and MARCO. They also express the class B scavenger receptors CD36 and CD163. Pharmacological and immunological blockade of class A and B scavenger receptors revealed a potential role for MARCO in the internalization of *C. sordellii*. Experiments with PMs revealed that MARCO, but not SR-AI/II, is important for macrophage ingestion of *C. sordellii*. These *in vitro* findings were supported by *in vivo* experiments documenting an important role for MARCO in intrauterine host defense against *C. sordellii* infection.
Conclusions: The CASR MARCO is critically important to the macrophage recognition of unopsonized *C. sordellii*.

Funding: This research was funded in part by National Institutes of Health Grants HD057176. Funding for the non-animal portions of this research provided by a Doris Duke Charitable Fund Clinical Scientist Development Award.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

GENETIC STUDIES OF VIRULENCE FACTORS IN THE CYTOTOXIC CLOSTRIDIA

Lyras, D.*
Department of Microbiology, Monash University, Victoria, Australia

The clostridia represent a diverse group of anaerobic spore-formers, many of which are animal and human pathogens. Genetic manipulation of these bacteria has proven to be a difficult task. Early work involved developing reliable and reproducible methods for the transformation of *C. perfringens* cells with recombinant plasmids with several well-characterised shuttle vectors being constructed during these studies. The successful use of these methods and vectors was, however, limited to a very small number of *C. perfringens* strains. The transformation methods developed for *C. perfringens* were not found to be successful for other clostridial species. We therefore developed a conjugation system based on the broad host-range plasmid RP4, using RP4-carrying *E. coli* donor strains to deliver recombinant plasmids into various clostridial recipients *via* conjugation. This method was successful for some *C. perfringens*, *C. difficile* and *C. septicum* strains but was variable and only worked for a limited number of strains. We have recently been able to expand the range of clostridial strains that we can genetically manipulate. By using a variety of different recombinant plasmids and donor strains we have now successfully and reproducibly manipulated *C. perfringens*, *C. chauvoei*, *C. septicum*, *C. sordellii* and epidemic human and animal *C. difficile* strains. The advent of this methodology has allowed us to confirm that the anti-sigma factor TcdC acts as a negative regulator of toxin production in *C. difficile* and to generate mutations in many animal and human *C. difficile* strains. In summary, in the past ten years dramatic progress has been made in the development of genetic methods for the analysis of a number of *Clostridium* species, to the extent that many of these pathogenic bacteria are now amenable to detailed and systematic genetic analysis. Our recent studies have now lead to a further advance in these techniques and should lead to a greater understanding of how these important pathogens cause disease.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

CONJUGATIVE PLASMIDS IN *CLOSTRIDIUM BOTULINUM*

Johnson, E.A.;* Marshall, K.M.; Johnson, E.A.
Department of Bacteriology, University of Wisconsin-Madison, Madison, WI USA

Clostridium botulinum produces seven distinct serotypes of the most lethal neurotoxins called botulinum neurotoxins (BoNTs). Five neurotoxin subtypes have been identified within serotypes A, B, E and F. The genes encoding different subtype neurotoxins of serotypes A, B and F were recently discovered to reside on large, highly homologous virulence plasmids in several proteolytic strains of *C. botulinum* including dual neurotoxin producing strains, suggesting that intra-species transfer of the toxin bearing plasmids may occur. To test this hypothesis, the virulence plasmids pCLK (strain Loch Maree), pCLJ (strain 657Ba), pCLL (strain 17B) and pBot706B (strain 706B) were 'tagged' with the erythromycin resistance gene (*ermB*) using the ClosTron mutagenesis system. The BoNT/A3 gene of pCLK and the BoNT/B gene of pCLJ, pCLL and pBot706B were inactivated by integration of the intron element containing the ErmB Retrotransposition-Activated Marker (RAM), resulting in the 'tagged' plasmids pCLK-Erm, pCLJ-Erm, pCLL-Erm and pBot706B-Erm. Transfer of the tagged plasmids to a nontoxigenic recipient *C. botulinum* strain (LNT01) was evaluated in mating experiments. The nontoxic *C. botulinum* strain LNT01 was chosen as the recipient because the presence of a tetracycline-encoded conjugative transposon Tn*916* in this strain provides an excellent means for the selection of transconjugants. The plasmid transfer to the transconjugants was confirmed by pulsed-field gel electrophoresis and Southern hybridization analyses. Transfer was shown to require cell-to-cell contact and was DNase resistant. This suggests that transfer of these plasmids occurred via a conjugation mechanism. This is the first evidence supporting conjugal transfer of four virulence plasmids in *C. botulinum*, and provides a probable mechanism for the widespread distribution of virulence plasmids in other *C. botulinum* strains. The potential transfer of *C. botulinum* virulence plasmids to other bacterial hosts in the environment or within the human intestine is of great concern for human pathogenicity and necessitates further characterization of these plasmids.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

REQUIREMENTS FOR THE GERMINATION OF *CLOSTRIDIUM SORDELLII* SPORES *IN VITRO*

Ramirez, N.; Abel-Santos, E.*
Department of Chemistry, University of Nevada, Las Vegas, Las Vegas, NV USA

Clostridium sordellii is a spore forming, obligate anaerobe, Gram-positive bacterium that can cause toxic shock syndrome after gynecological procedures. Although the incidence of *C. sordellii* infection is low, it is fatal in most cases. Since spore germination is believed to be the first step in the establishment of *Bacilli* and *Clostridia* infections, we analyzed the requirements for *C. sordellii* spore germination *in vitro*. Our data showed that *C. sordellii* spores require three structurally different amino acids and bicarbonate for maximum germination. Contrary to *Bacilli* species, D-alanine had no effect on *C. sordellii* spore germination. *C. sordellii* spores only germinated in a narrow pH range between 5.7 and 6.5. In contrast, *C. sordellii* spore germination was significantly less sensitive to temperature changes as compared to the *Bacilli*. Analysis of the kinetics of *C. sordellii* spore germination showed strong allosteric behavior in the binding of L-phenylalanine and L-alanine, but not bicarbonate or L-arginine. By comparing germinant apparent binding affinities with their known *in vivo* concentrations, we postulated a mechanism for differential *C. sordellii* spore activation in the female reproductive tract.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

FINDING THE GENES FOR PLASMALOGEN BIOSYNTHESIS IN CLOSTRIDIA

Goldfine, H.;*[1] Turkarslan, S.;[2] Daldal, F.[2]
[1]Department of Microbiology, School of Medicine, and
[2]Department of Biology, University of Pennsylvania, Philadelphia, PA USA

Most clostridia that have been examined including *Clostridium perfringens, C. tetani*, *C. botulinum, C. novyi* and *C. sporogenes* contain plasmalogens, 1-O-alk-1'enyl-2-acyl glycerophospholipids. In anaerobes unlike animals, the biosynthesis of plasmalogens does not require molecular oxygen. At present the genes and the pathway for plasmalogen biosynthesis in clostridia are unknown. We have taken bioinformatic and genomic approaches to the discovery of these genes. We identified approximately 100 candidate genes by comparing the genomes of clostridia that have plasmalogens with those of other Gram positive bacteria that do not. In addition we have found that C. *tetani* E88 strain, which has had its genome sequenced, does not contain plasmalogens whereas all other C. *tetani* strains we examined do. We have obtained the complete genome sequences of two of the strains that have plasmalogens and have compared the amino acid sequences encoded by genes identified by bioinformatics with the amino acid sequences of the same genes in the three strains. By this means we have identified six genes found in clostridia, but not in aerobic or facultative organisms as candidate genes. These will be mutated to test for effects on plasmalogen biosynthesis in clostridia. Plasmalogens in anaerobes have presumably been retained for a large part of the history of life on earth. Although laboratory strains grow well without them, they apparently are important for life outside the laboratory. The ability to mutate genes in this pathway will enable us to study the importance of these lipids in the life of clostridia and for the pathogenic potential of disease-causing organisms.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

TWO CASES OF BOTULISM DUE TO *CLOSTRIDIUM BARATII* PRODUCTION OF TYPE F BOTULISM TOXIN

Hannett, G.E.;*[1] Schaffzin, J.K.;[2] Davis, S.W.;[1] Fage, M.P.;[2] Johnson, G.S.;[2] Schoonmaker-Bopp, D.;[1] Dumas, N.;[1] Musser, K.[1]
[1]Wadsworth Center, New York State Department of Health, Albany, NY USA
[2]Bureau of Communicable Disease Control, New York State Department of Health, Albany, NY USA

Background: In the United States, botulism due to type F toxin is an extremely rare occurrence, with an average of less than one documented case per year. In cases where type F botulism toxin has been laboratory confirmed, *Clostridium baratii* is usually the etiologic agent. In 2007, 2 laboratory confirmed cases of type F botulism occurred within 8 days of each other in patients that lived only 23 miles apart. We report on the epidemiologic and laboratory investigation of those cases.
Methods: Mouse bioassay for botulism toxin was performed on serum, stool, and isolates. Stools were tested by PCR for detection of the gene responsible for type F toxin production (*bont/F)*, and by culture. Twenty food and 6 environmental samples were collected from the households and tested by PCR and culture. Isolates of *C. baratii* obtained from the patient specimens were typed using pulsed-field gel electrophoresis (PFGE). An epidemiologic investigation was conducted to identify a source common to the 2 cases.
Results: *C. botulinum* type F toxin was demonstrated in the stool from patient A, and the serum from patient B. *C. botulinum* toxin, type indeterminate, was demonstrated in the serum from patient A. *C. baratii* that produced type F botulism toxin was isolated from the stool of both patients. PCR performed on the stools from both patients was positive for the presence of *C. baratii bont/F.* PCR and culture on all food and environmental samples was negative. Both patients were hospitalized and had clinical courses consistent with type F botulism. An epidemiologic exposure common to the 2 patients could not be identified. PFGE patterns of the 2 patient isolates differed by >7 bands, indicating different strains.
Conclusion: *C. baratii* that produces botulism toxin is sporadic in occurrence. Despite clustering in time and space, these cases appear to be unrelated. PCR, culture, and subsequent strain typing can be of great assistance in the investigation of botulism cases.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

CARBON METABOLISM ANALYSIS OF IMPORTANT *CLOSTRIDIUM* SPECIES

Lei, X.H.;* Bochner, B.R.
Biolog, Inc., Hayward, CA USA

Background: *C. difficile* is a major human pathogenic *Clostridium* species. *C. sordellii* and, more recently, *C. bolteae* have also been implicated in serious human infections. It is of fundamental importance to understand the basic metabolic phenotypes of these pathogens. Using our previously developed high throughput technology, Phenotype MicroArray (PM), that allows thousands of phenotypes to be tested simultaneously and automatically, we here demonstrate similarities and differences of type strains of *C. difficile*, *C. sordellii*, and *C. bolteae* on ~200 carbon metabolism assays, to add to our understanding of these human pathogens.
Methods: PM plates and all materials were deoxygenated prior to experiments. Cells were cultured for 24 hours on Biolog Universal Anaerobe agar with 5% Sheep's Blood prior to PM plate inoculation. All handling of cells was in an anaerobic atmosphere (5% H_2, 5% CO_2, 90% N_2) and PMs were incubated inside sealed O_2-impermeable and transparent plastic bags in the OmniLog® instrument for 24 hours, which automatically collects kinetic data once every 15 minutes on each array well into computer files for analysis by OmniLog PM software.
Results: *C. difficile* can utilize six-carbon sugars (e.g., D-glucose, D-fructose, D-galactose, D-mannose) and five-carbon sugars (e.g., D-ribose, D-xylose) as sole carbon sources. It also uses N-acetyl-D-glucosamine, but can not use amino acids as a sole carbon source except for D- and L-serine. In contrast, *C. bolteae* uses not only the carbon sources that *C. difficile* can but also some that *C. difficile* can not, such as D-sorbitol, D-gluconic acid, L-galactonic acid-γ-lactone, α-methyl-D-galactoside, N-acetyl-neuraminic acid, maltose, sucrose, D-melibiose, D-melezitose, maltotriose, D-raffinose, dextrin, stachyose, tween compounds, and nucleosides. *C. sordellii* is more active in utilizing different carbon sources than *C. difficile*, but less active than *C. bolteae*. Interestingly, however, it shows a stronger ability than *C. bolteae* and *C. difficile* to utilize α-keto-butyric acid, D- and L-serine, and it also uses D- and L-threonine as a carbon source, which can not be utilized by the other two.
Conclusions: PM technology provides the first detailed comparative overview of *C. difficile*, *C. sordellii*, and *C. bolteae* carbon metabolism with nearly 200 carbon sources tested. *C. bolteae* is the most active and versatile in carbon metabolism among the three, and *C. difficile* is least active with a narrower spectrum of usable carbon sources. All three have limited ability to utilize amino acids as carbon source. We plan to expand the number of species and PM panels tested in future studies.

Anaerobe 2010

The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

COMPARISON OF VACUOLATING & LETHAL ACTIVITIES OF SIX REFERENCE ATCC STRAIN OF *CLOSTRIDIUM HISTOPLYTICUM*

Jankowska-Steifer, E.;[1] Ekiel, A.;[2] Komar, A.;[1] Wiechuła, B.;[2] Jóźwiak, J.;[2] Moskalewski, S.;[2] Martirosian, G.*[1,2]
[1]Department of Histology and Embryology, Medical University of Warsaw, Warsaw, Poland
[2]Department of Medical Microbiology, Medical University of Silesia, Katowice, Poland

We have previously demonstrated that *C. histolyricum* reference strain ATCC 19401 produces not only lethal factor but also hitherto unrecognized vacuolating toxin. The aim of this study was to compare vacuolating, lethal, protease, collagenase and clostripain activities of six reference strains of *C. histolyticum* (ATCC 6282, 8034, 17859, 17860, 19401 and 25770). All strains elaborated vacuolating and lethal toxins as well as collagenase, clostripain and proteases, but with different yield. Strain ATCC 19401 demonstrated considerable vacuolating and lethal activities and low activity of collagenase, clostripain and proteases. Strain ATCC 17860 displayed high activity of proteases and both toxins , and low collagenase and clostripain activity. Thus, correlation between vacuolating and lethal toxins and enzymatic activities of particular reference strain of *C. histolyticum* was not evident.

This work was supported by a grant from the Polish Ministry of Scientific Research and Information Technology Nr 40404132/0949.

Anaerobe 2010
The 10th Biennial Congress of the
Anaerobe Society of the Americas

Philadelphia, PA USA • July 7-10, 2010

AN UPDATE ON CYTOTOXIC CLOSTRIDIAL PATHOGENS

DEVELOPMENT OF A ZEBRAFISH (*DANIO RERIO*) MODEL TO SCREEN FOR NOVEL EXOTOXINS PRODUCED BY *CLOSTRIDIUM* SPP. ISOLATED FROM THE HUMAN GUT

Schroeter, K.;* Lum, W.; Van Raay, T.; Allen-Vercoe, E.
Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON Canada

Species belonging to the Clostridiales order are dominant members of the human gut microflora, and several species are known to be important pathogens of humans. Most clostridial pathogens secrete potent exotoxins which are central to their virulence and, as a group, exert wide-ranging effects on target host cells. Recent studies have indicated a positive correlation between certain gut-derived *Clostridium* spp. antigens and inflammatory bowel disease (IBD). Additionally, overgrowth of certain gut clostridial species has been found to be associated with some forms of autism. We hypothesize that a subpopulation of gut associated Clostridiales species are potentially toxigenic, with important consequences to human health during episodes of microflora imbalance (dysbiosis).

Detection of novel bacterial toxins is challenging, since many of these toxins share commonality in their mechanisms of action, but their target sites and encoding genes may be diverse. We developed a high-throughput screening strategy for novel bacterial (and specifically clostridial) toxins using a well-characterized vertebrate model, the zebrafish (*Danio rerio*) embryo.

Zebrafish embryos are ideally suited to toxicological screening because of their rapid and well-defined development, small size, transparency and ease of maintenance. We used a known toxigenic *Clostridium* sp., *C. difficile*, to demonstrate the feasibility of applying bacterial culture supernatants directly to zebrafish embryos in a 96-well plate, high-throughput manner and determining a pathologic response.

Culture supernatant from *wt C. difficile* strain MOH9W applied to zebrafish embryos at the shield stage of development induced dose-dependent, reproducible pathology including shortened jaws, reduced blood flow, blood accumulation in the heart cavity and cardiac arrhythmia. Treatment with a commercially available preparation of *C. difficile* toxins TcdA and TcdB also gave rise to dose-dependent pathology, although the range and severity of defects was not as marked as those seen from culture supernatant preparations. This in turn suggests that the secretion of toxins additional to TcdA and TcdB act in concert to enhance pathogenicity in this model.

Our results validate the use of zebrafish embryos in high-throughput screening for novel clostridial (or other bacterial) toxins. We are currently applying this model to screen a selection of clostridial isolates from the GI tracts of patients with IBD or late-onset autism.