Anaerobe 16 (2010) 155–160

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Pathogenesis and Toxins

Lethal toxin is a critical determinant of rapid mortality in rodent models of *Clostridium sordellii* endometritis

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ARTICLE INFO

Article history:
Received 14 February 2009
Received in revised form
16 May 2009
Accepted 4 June 2009
Available online 13 June 2009

Keywords:
Clostridium infection
Endometritis
Toxic shock syndrome
Reproductive tract infections
Anaerobes

ABSTRACT

The toxigenic anaerobe *Clostridium sordellii* is an uncommon but highly lethal cause of human infection and toxic shock syndrome, yet few studies have addressed its pathogenetic mechanisms. To better characterize the microbial determinants of rapid death from infection both *in vitro* and *in vivo* studies were performed to compare a clinical strain of *C. sordellii* (DA-108), isolated from a patient who survived a disseminated infection unaccompanied by toxic shock syndrome, to a virulent reference strain (ATCC9714). Rodent models of endometrial and peritoneal infection with *C. sordellii* ATCC9714 were rapidly lethal, while infections with DA-108 were not. Extensive genetic and functional comparisons of virulence factor and toxin expression between these two bacterial strains yielded many similarities, with the noted exception that strain DA-108 lacked the *tcsL* gene, which encodes the large clostridial glucosyltransferase enzyme lethal toxin (TcsL). The targeted removal by immunoprecipitation of TcsL protected animals from death following injection of crude culture supernatants from strain ATCC9714. Injections of a monoclonal anti-TcsL IgG protected animals from death during *C. sordellii* ATCC9714 infection, suggesting that such an approach might improve the treatment of patients with *C. sordellii*-induced toxic shock syndrome.

1. Introduction

Clostridium sordellii is a soil-dwelling, anaerobic, spore-forming Gram-positive bacillus that uncommonly causes infections in humans and animals [1]. Diseases caused by *C. sordellii* include bloodstream and necrotizing soft tissue infections associated with black tar heroin use [2–4]; uterine infections following childbirth or spontaneous/medical abortion [5,6]; and soft tissue infections associated with contaminated cadaveric orthopaedic graft material [7]. The mortality of these infections is high, approaching 70% [1] and varies with the clinical presentation. Although rare, *C. sordellii* infections attract attention because of their high mortality and propensity to present with a characteristic "toxic shock syndrome." This stereotypical syndrome includes the sudden onset of weakness, nausea, and vomiting; progressive and refractory hypotension; local and spreading edema; severe hemoconcentration; and a marked leukemoid reaction [8].

C. sordellii infections are remarkable for their heterogeneity in both clinical presentation and outcome. While some patients present (and die) with refractory toxic shock despite harboring a localized infection of the soft tissues [9,10], other patients have local or disseminated infections in the absence of this stereotypical syndrome [11,12]. The degree of tissue necrosis may also vary among patients with *C. sordellii* disease, despite similar modes of infection [2,13,14]. The clinical manifestations of *C. sordellii* infection are theorized to derive primarily from a diverse array of secreted toxins, including a cholesterol-dependent cytotoxin/hemolysin (*a.k.a.* sordellilysin) [14]; phospholipase C (lecithinase) [15]; neuraminidase (sialidase) [16]; DNAse [17]; hyaluronidase [1]; collagenase [17]; and two members of the large clostridial cytotoxin class of toxins, hemorrhagic toxin (TcsH) and lethal toxin (TcsL) [1]. The role each of these individual toxins plays in the pathogenesis of human infection remains incompletely defined. The expression of these toxins by clinical isolates of *C. sordellii* is highly variable [14,18]. Some have postulated that different "virotypes" of *C. sordellii* (expressing distinct virulence factor profiles)

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doi:10.1016/j.anaerobe.2009.06.002

might explain the marked variability in clinical presentation during infection [14].

A clinical strain of *C. sordellii* was isolated from a patient who survived postpartum *C. sordellii* endometritis and bacteremia that was not complicated by toxic shock syndrome. We questioned whether the *C. sordellii* strain isolated from this patient was deficient in TcsL production, which might explain the absence of toxic shock and rapid death. We attempted to answer this question using both *in vitro* comparative analyses with a TcsL-positive reference strain of *C. sordellii* (ATCC9714) and *in vivo* experiments. A new mouse model of *C. sordellii* endometritis was developed for these studies. Our results, for the first time, directly correlate the expression of virulence factors by a clinical isolate of *C. sordellii* with the outcome of infection in a human host and in animal models of disease. These data shed new light on the nature of *C. sordellii* pathogenesis and might facilitate the development of preventive or therapeutic strategies against this deadly infection.

2. Materials and methods

2.1. Animals

125–150 g female Wistar rats were obtained from Charles River Laboratories (Portage, MI), 3–6-week-old female C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME), and 8–10-week-old female 129 SvEv mice from Taconic (Hudson, NY). Animals were treated per NIH guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

2.2. Reagents

Reinforced clostridial medium (RCM) and brain heart infusion broth were from BD Biosciences (San Jose, CA). A mouse monoclonal anti-TcsL IgG1 (lot CSORN A3B2-3B8) was prepared in a bioreactor as previously described [19]. This IgG protects mice from death from TcsL injection [19]. *C. sordellii* polyclonal antitoxin IRP 501(04) containing 170 antitoxin units per mL (AU/mL), was produced in goats in August 2004 using *C. sordellii* strain 7502-1-11 (obtained originally from Montana State University, Bozeman, MT, on September 16, 1968) and standardized against the World Health Organization gas-gangrene (*C. sordellii*) international antitoxin, equine origin. The *C. sordellii* strain 7502-1-11 is toxic to mice and expresses TcsL but does not express TcsH (data not shown). Goat polyclonal IgG against *Clostridium difficile* toxin A was from EMD Chemicals, Inc (Gibbstown, NJ).

2.3. Bacteria

C. sordellii strain ATCC9714 was from the American Type Culture Collection (Manassas, VA). The clinical strain DA-108 was isolated from the blood of a patient who survived postpartum endometritis. It was verified to be *C. sordellii* by PCR amplification of a *C. sordellii*-specific portion of the 16S rRNA gene (not shown; see Table 1 for primer pairs). Bacteria were grown in broth overnight at 37 °C in RCM in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI).

2.4. Toxin production

For *in vivo* experiments, crude toxin preparations were made using 10 ml of overnight broth cultures of *C. sordellii*. Cultures were subjected to centrifugation (3000 RPM, 10 min, 22 °C). The supernatant was filtered (0.22 μm) into Amicon Ultra-4 centrifugal filters with a 100 kDa molecular weight cut off (Millipore, Billerica, MA). Amicons were centrifuged (5000 × *g*, 20 min, 22 °C) and the ~100-fold concentrated retentate stored at −20 °C. For immunoblot analyses, neuraminidase assays, and TcsH assays (see below), concentrated toxins from ATCC9714 and DA-108 were prepared as follows. Bacteria were grown in anaerobic meat medium for 72 h then inoculated into brain heart infusion dialysis flasks (1L trypsin flasks). After anaerobic incubation (37 °C, 72 h), cells were removed by centrifugation (7000 RPM × 60 min). A protease inhibitor cocktail (Sigma) was added to 0.22 μm-filtered culture supernatant. Toxins were concentrated with acetone. Ice cold supernatants were re-filtered through 0.45 μm pore polycarbonate filters and acetone (−20 °C) was added slowly with stirring to a final concentration of 5:1 (vol/vol) acetone: supernatant. 200 ml was concentrated and resuspended in 20 ml of 0.01 M PBS. Toxin was aliquoted and stored at −80°C until further use.

2.5. Phospholipase C and neuraminidase assays

Phospholipase C activity was assayed according to a previously published protocol [20] using toxin samples (100 μl) prepared from overnight cultures as above. Neuraminidase (sialidase) activity was determined using *C. sordellii* toxins, prepared as above, with a commercially available neuraminidase activity kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). *Clostridium perfringens* neuraminidase was included as a positive control.

2.6. Pleural effusion volume measurement

C57BL/6J mice were infected intrauterine (i.u.) with 1×10^7 CFU of ATCC9714 or DA-108 bacteria. At the immediate time of death (ATCC9714-infected mice) or after 48 h (DA-108-infected mice) the volume of intrathoracic (bilateral pleural space) fluid was measured, then compared with that of uninfected mice.

2.7. Intrauterine infection, intraperitoneal infection, and intoxication

Intrauterine infection was performed in rats or C57BL/6J mice according to a previously published protocol [21]. Mice were given an intraperitoneal (i.p.) inoculation with live *C. sordellii* in 100 μl of sterile PBS. In other experiments mice were injected i.p. with 100 μl of sterile PBS containing a 1:5 dilution of crude *C. sordellii* toxins prepared as above (20 μl toxin mixed with 80 μl PBS).

2.8. Administration of antitoxin or anti-TcsL immunoglobulin in vivo

Rats were infected i.u. with *C. sordellii* ATCC9714 (1×10^{10} CFU) in 100 μl containing 60 μl of antitoxin or anti-TcsL IgG. Rats receiving antitoxin were treated daily with i.p. injections of antitoxin (300 μl) on days 1, 2, and 3 post-infection while animals given anti-TcsL IgG subsequently were treated with i.p. injections of anti-TcsL IgG (300 μl twice daily) on days 1, 2, and 3 post-infection. C57BL/6 mice were infected i.u. with *C. sordellii* ATCC9714 (1×10^6 CFU) in 25 μl of anti-TcsL IgG. Mice were subsequently treated with i.p. injections of anti-TcsL IgG (75 μl twice daily) on days 1, 2, and 3 post-infection.

2.9. Immunoprecipitation of TcsL from C. sordellii ATCC9714 toxins

To remove TcsL from *C. sordellii* ATCC9714 filtered culture supernatants, 100 μl of filtered supernatant was added to 200 μl of sterile PBS, then 20 μl of the monoclonal anti-TcsL IgG was added.