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A novel murine model of *Clostridium sordellii* myonecrosis: Insights into the pathogenesis of disease

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ABSTRACT

Clostridium sordellii infections have been reported in women following natural childbirth and spontaneous or medically-induced abortion, injection drug users and patients with trauma. Death is rapid and mortality ranges from 70 to 100%. Clinical features include an extreme leukemoid reaction, the absence of fever, and only minimal pain or erythema at the infected site. In the current study, we developed a murine model of *C. sordellii* soft tissue infection to elucidate the pathogenic mechanisms. Mice received 0.5, 1.0 or 2.0 $\times$ 10^6 CFU *C. sordellii* (ATCC 9714 type strain) in the right thigh muscle. All doses caused fatal infection characterized by intense swelling of the infected limb but no erythema or visible perfusion deficits. Survival rates and time to death were inoculum dose-dependent. Mice developed a granulocytic leukocytosis with left shift, the onset of which directly correlated with disease severity. Histopathology of infected tissue showed widespread edema, moderate muscle damage and minimal neutrophil infiltration. Circulating levels of granulocyte colony-stimulating factor (G-CSF), soluble tumor necrosis factor receptor I (sTNF-RI) and interlukin-6 (IL-6) were significantly increased in infected animals, while TNF-α, and IL-1β levels were only mildly elevated, suggesting these host factors likely mediate the leukocytosis and innate immune dysfunction characteristic of this infection. Thus, this model mimics many of the salient features of this infection in humans and has allowed us to identify novel targets for intervention.
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1. Introduction

Clostridium sordellii causes a rapidly progressive soft tissue infection [1–3]. Our recent review of 45 *C. sordellii* cases demonstrated an overall mortality of 70% [1]. Of the fatal infections, 74% occurred in women undergoing childbirth or spontaneous or induced abortions and mortality was 100%. *C. sordellii* infections associated with traumatic wounds, injecting drug use or surgery were fatal in 52% of cases [1]. Initial symptoms included nausea, dizziness, lethargy, and mild tenderness at sites of infection. Most patients (73%) were afebrile. Within hours of presentation, most developed a unique constellation of clinical features including refractory hypotension, severe tachycardia, profound capillary leak syndrome with hemoconcentration. Development of an extreme leukemoid reaction (LR: 75-200 x 10^3 cells/μL whole blood) with a left shift was the sole predictor of a fatal outcome [1]. Though the development of an extreme LR is pathognomonic for *C. sordellii* infections and portends fatal outcome, the mechanisms remain entirely unknown.

The virulence of *C. sordellii* is strain variable and reflects the profile of exotoxins produced by individual organisms. Pathogenic strains of *C. sordellii* produce up to seven known exotoxins [4]. Of these, the hemorrhagic toxin (HcsT) and lethal toxin (LcsT) are thought to mediate the pathogenesis of infection [4]. Other toxins include a cholesterol-dependent hemolysin, phospholipase C, neuraminidase, DNAse, hyaluronidase, and collagenase. However, the specific roles of these auxiliary exotoxins during infection remain to be clearly defined.

Animal models are extremely important to elucidating the mechanisms associated with disease pathogenesis, dynamics of the host response and the specific roles of bacterial toxins during infection. A murine intrauterine model of *C. sordellii* infection has been developed to investigate *C. sordellii*-associated endometritis [5]. In addition, experimental fatal toxic shock syndrome studies have utilized intraperitoneal injections of the lethal toxin of *C.*

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sordellii in mice and rats [6]. However, an *in vivo* model to investigate *C. sordellii* intramuscular infections (IM) has yet to be established, despite the fact that soft tissue infections represent nearly one-third of all reported cases in humans [1].

The goals of the present work were to establish the first murine model of *C. sordellii* muscle infection that mimics the salient features of this infection in humans and to use this model to investigate the mechanisms driving the extreme *C. sordellii* LR. Mice challenged intramuscularly with a clinically relevant strain of *C. sordellii* developed signs and symptoms comparable to those associated with human infection, including development of an LR, and had a rapidly lethal outcome. Reduced circulating TNFα and IL-1β levels and marked increases in sTNF-RI, G-CSF and IL-6 provide the first-ever clues as to the mechanisms responsible for the characteristic absence of fever and extreme leukemoid reaction observed in humans with *C. sordellii* infection. These findings demonstrate the relevance and utility of this model of *C. sordellii* infection in elucidating the mechanisms responsible for the local and systemic effects of infection with this important human pathogen and suggest new interventional targets.

2. Materials and methods

2.1. Bacteria

C. sordellii type strain #9714 (American Type Culture Collection [ATCC], Manassas, VA) was used. This strain was originally isolated from an acute human soft tissue infection [4]. A Bactron II anaerobic chamber (Sheldon Manufacturing, Cornelius, OR) was utilized to maintain an anaerobic environment for *C. sordellii* growth and manipulation.

2.2. Bacterial cultivation and inoculum preparation

Stock cultures were streaked on Brucella blood agar plates (Anaerobe Systems, Morgan Hill, CA) and a single isolated colony was used to inoculate Brain Heart Infusion (BHI; Becton Dickinson, San Jose, CA) broth. Cultures were grown anaerobically overnight at 37 °C. The following morning, 1% of the overnight culture was used to inoculate 250 mL of fresh, pre-reduced BHI liquid media. This culture was grown to an OD_{600} of approximately 1.7 (late-stationary phase culture). Organisms were collected by centrifugation (5000 × *g*, 15 min, 4 °C), washed twice and resuspended in pre-chilled saline. A working stock of 2.0×10^7 colony forming units (CFU)/mL was prepared based on a previously determined relationship between CFU and OD_{600}. The working stock was serially diluted 2-fold in saline to achieve desired concentrations. Bacterial concentrations of these preparations were confirmed by plating serially diluted samples in duplicate on BHI agar plates. Plates were incubated anaerobically at 37 °C and colonies counted the following day.

2.3. Experimental C. sordellii infection

All animal experiments were approved by the Boise, ID Veterans Affairs Medical Center's Institutional Animal Care and Use Committee and adhered to guidelines of the National Institutes of Health. Adult female C57BL/6 mice (20/group) were injected intramuscularly in the right upper thigh with 0.5, 1.0 or 2.0×10^6 CFU (low, medium and high doses, respectively) of *C. sordellii* ATCC 9714. These concentrations were selected based on similar studies previously performed by our group (data not published). Immediately following challenge, infected animals were randomly divided into 'survival' and 'pathogenesis' subgroups. The survival subgroup was closely monitored over 5 days and mortalities noted. In the pathogenesis subgroup, blood specimens were taken from 2 animals/time point and infected tissues were examined for histologic abnormalities as described below.

2.4. Histopathological analysis

Two mice per inoculum group from the 'pathogenesis' subgroups were randomly selected at 12, 21, 30, 39 and 48 h after infection and a single blood specimen was obtained by a retro-orbital draw from isoflurane-anesthetized animals to determine circulating white blood cell counts and differentials (see below). Animals were then sacrificed by cervical dislocation and the infected muscle tissue (and the contra-lateral non-infected control muscle) was surgically removed and fixed in 10% neutral buffered formalin. Formalin-fixed tissues were embedded in paraffin, stained with hematoxylin-eosin and examined and scored by a blinded pathologist for evidence of; 1) inflammation, 2) edema, 3) tissue necrosis, and 4) hemorrhage, on a scale of 0–3 as follows; 0: none, 1: mild, 2: moderate, and 3: marked.

2.5. White blood cell (WBC) counts and differentials

WBC counts were determined in two animals per time point by adding 10 μL of each whole blood specimen to 180 μL of Turks stain solution (1:20 dilution). Each sample was counted twice by hemocytometer, and the final WBC count is given as the average of these two counts. For WBC differential counts, cytospin samples were prepared. Here, 10 μL of each whole blood specimen was added to 0.5 mL of PharM Lyse (BD Biosciences, San Jose, CA) to lyse red blood cells. Samples were zap vortexed, incubated at room temperature for 15 min, and spun at 300 × *g* for 5 min. Overlaying supernatants were aspirated and the remaining WBC pellets resuspended in 500 μL of PBS +7% culture grade bovine serum albumin (BSA). 450 μL of this material were then added to individual chambers of a Shandon Cytospin 2 centrifuge (Block Scientific, Inc, Bohemia, NY) and spun at 200 × *g* for 10 min. After air drying, slides were stained via an Aerospray 7120 hematology automatic slide stainer-cytocentrifuge (Wescore, Inc, Logan, UT) and sent to the University of Idaho Animal and Veterinary Science Department (Caldwell, ID) where WBC differentials were determined by a blinded observer with expertise in this area. Cells were counted using an Olympus CX31 microscope (1000x magnification). 100 cells were counted in two separate fields, and data are reported as the average of these 2 counts. Population ratios for polymorphonuclear leukocytes, lymphocytes, monocytes, eosinophils, band cells, and metamyelocyte/myelocyte cells were determined.

2.6. Murine protein cytokine array

Relative serum levels of 30 cytokines and chemokines were measured by a custom slide-based mouse cytokine array (RayBiotech, Norcross, GA) according to the manufacturer's instructions. Briefly, the arrays were blocked with the supplied blocking buffer for 30 min, then incubated with 100 μl of serum collected from *C. sordellii*-infected or no treatment control animals (diluted 1:10 with blocking buffer) at 25 °C for 2 h. After washing, arrays were incubated with biotin-conjugated primary antibody and horseradish peroxidase-conjugated streptavidin. Arrays were sent to RayBiotech where a semi-quantitative analysis of the comparative intensity of the spots was performed. The relative intensities of each cytokine were normalized to control spots on the same array. The final cytokine values were calculated by subtracting control animal measurements from those obtained from infected animals. Cytokines/chemokins tested were: Cluster of Differentiation (CD) 30 ligand (CD30L), CD40, Fas Ligand, granulocyte colony-