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Original article

The role of complement opsonization in interactions between *F. tularensis* subsp. *novicida* and human neutrophils

Jason H. Barker [a,*], Ramona L. McCaffrey [a], Nicki K. Baman [a], Lee-Ann H. Allen [a,b], Jerrold P. Weiss [a,b], William M. Nauseef [a,b]

[a] *Inflammation Program and Department of Medicine, Veterans Affairs Medical Center and the University of Iowa, 2501 Crosspark Road, D168-MTF, Coralville, IA 52241, USA*

[b] *Department of Microbiology, Veterans Affairs Medical Center and the University of Iowa, 2501 Crosspark Road, D168-MTF, Coralville, IA 52241, USA*

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Abstract

The remarkable infectiousness of *Francisella tularensis* suggests that the bacterium efficiently evades innate immune responses that typically protect the host during its continuous exposure to environmental and commensal microbes. In our studies of the innate immune response to *F. tularensis*, we have observed that, unlike the live vaccine strain (LVS) of *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *novicida* U112 opsonized in pooled human serum activated the NADPH oxidase when incubated with human neutrophils. Given previous observations that *F. tularensis* fixes relatively small quantities of complement component C3 during incubation in human serum and the importance of C3 to neutrophil phagocytosis, we hypothesized that *F. tularensis* subsp. *novicida* may fix C3 in human serum more readily than would LVS. We now report that *F. tularensis* subsp. *novicida* fixed approximately six-fold more C3 than did LVS when incubated in 50% pooled human serum and that this complement opsonization was antibody-mediated. Furthermore, antibody-mediated C3 deposition enhanced bacterial uptake and was indispensable for the neutrophil oxidative response to *F. tularensis* subsp. *novicida*. Taken together, our results reveal important differences between these two strains of *F. tularensis* and may, in part, explain the low virulence of *F. tularensis* subsp. *novicida* for humans.
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Keywords: *Francisella tularensis*; Neutrophils; Complement activation; complement C3; Humans; Phagocytosis; Respiratory burst

1. Introduction

Francisella tularensis is a small Gram-negative coccobacillus that causes tularemia, a highly infectious and life-threatening zoonosis of humans. Infection can be acquired via aerosol, insect bites, or inoculation onto broken skin or mucous membranes, and infections are typically seen in people with exposure to infected animals or contaminated soil or water [1]. The low infectious dose and the high mortality of pneumonic tularemia sparked interest in the use of the organism as an agent of biowarfare, leading to its weaponization during the Cold War [2]. Consequently, *F. tularensis* has been deemed a category A agent of bioterrorism and a priority for research.

F. tularensis is divided into several subspecies. *F. tularensis* subsp. *tularensis* is most virulent to humans and is found in North America, whereas subsp. *holarctica* is less virulent and can be found throughout the Northern Hemisphere [1]. Two other subspecies, *novicida* and *mediasiatica*, rarely cause disease in humans. Given the virulence and infectiousness of the wild-type *tularensis* and *holarctica* strains, much of the existing research has been performed using an attenuated live vaccine strain (LVS, derived from *F. tularensis* subsp. *holarctica*) and subsp. *novicida* (FtN), both of which cause a lethal invasive infection of mice [3]. They share with the more virulent strains of a *Francisella* pathogenicity island and the ability to replicate within macrophages of many species, including humans [1], but the basis for their decreased

* Corresponding author. Tel.: +1 319 335 4594; fax: +1 319 335 4194.
E-mail address: jason-barker@uiowa.edu (J.H. Barker).

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virulence is currently unclear. Furthermore, *F. tularensis* subsp. *novicida* is distinct antigenically from subsp. *tularensis* and subsp. *holarctica* [4] and apparently lacks a capsule-like material that the other subspecies possess ([5] and M.A. Apicella, unpublished observations). Thus, although the various subspecies are similar genetically and share some virulence factors, differences remain that are sufficient to render some strains highly virulent for humans whereas others rarely cause disease. The determinants of these differences in virulence are largely undefined.

Previous studies from our group have shown that LVS fails to stimulate the oxidative burst in human neutrophils (polymorphonuclear leukocytes, PMN) [6]. Intriguingly, we have observed that unlike LVS, *F. tularensis* subsp. *novicida* (FtN) stimulates the PMN NADPH oxidase. It has been reported that LVS fixes small quantities of complement component C3 to its surface during incubation in human serum [7], and given the importance of complement opsonization to PMN phagocytosis [8,9], we hypothesized that FtN would fix more C3 than does LVS when incubated in pooled human serum (PHS). We further hypothesized that uptake of FtN and the subsequent PMN oxidative burst would be dependent upon this increased C3 deposition.

2. Materials and methods

2.1. Materials

Endotoxin-free phosphate buffered saline (PBS) and Hank's buffered salt solution (HBSS) with or without divalent cations were from Mediatech, Inc. (Herndon, VA). Endotoxin-free water, normal saline, and human serum albumin were from Baxter International Inc. (Deerfield, IL). Bovine serum albumin (BSA), luminol, and 10% neutral buffered formalin were from Sigma–Aldrich Corp. (St. Louis, MO). Clinical grade dextran was from Pharmacosmos (Holbaek, Denmark). Difco cysteine heart agar (CHA) was from Becton Dickinson (Franklin Lakes, NJ). Sensitized sheep erythrocytes, gelatin veronal buffer, and purified human complement component C3 were from Complement Technology, Inc. (Tyler, TX). Fluorescent labeled secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

2.2. Human sera

Pooled human serum (PHS) was obtained from approximately 10 healthy donors without a history of tularemia. Immune serum from four patients diagnosed with tularemia was the gift of Dr. Gregory Storch (Washington University School of Medicine, St. Louis, MO). Immune sera were heat-inactivated (at 56 °C for 30 min) and pooled with a resulting agglutinating titer of 1:640, determined as previously described with slight modification [10]. Serum from a patient with agammaglobulinemia was the gift of Dr. Peter Densen, University of Iowa, Iowa City, IA. Functional activity of the classical pathway of complement (CH50) was assessed as described previously [11]. For some experiments, aliquots of PHS were depleted of antibody to FtN. Formalin-treated FtN (3×10^9/ml) was incubated in PHS for 30 min on ice. Bacteria were centrifuged and incubation with bacteria was repeated. Aliquots of serum were frozen at −80 °C until use.

2.3. PMN isolation

PMN were isolated from healthy donors as described previously using dextran sedimentation followed by Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) density-gradient separation [12].

2.4. Bacteria

F. tularensis subsp. *holarctica* strain LVS (ATCC strain 29684) was obtained from Dr. Michael Apicella, University of Iowa and *F. tularensis* subsp. *novicida* U112 was obtained from Dr. Colin Manoil at the University of Washington (www.francisella.org). LVS and FtN were grown for 48 and 24 h, respectively, on CHA/9% sheep blood plates at 37 °C in 5% CO_2. Bacteria were formalin fixed in 10% buffered formalin for 30 min. Periodate treatment was carried out as previously described in Ref. [13].

2.5. Opsonization

Washed francisellae (1.2×10^9 bacteria/ml) were suspended in 50% PHS, incubated with mixing at 37 °C, and washed twice. Incubation in serum did not affect the viability of the *Francisella* strains used in this study (not shown). Pretreatment of bacteria in immune serum was carried out for 30 min at 37 °C at a dilution of immune serum that did not result in agglutination (1:600–1:1000). Bacteria were pelleted and resuspended in 50% pooled PHS as a source of complement and incubated at 37 °C for 30 min as described above. In some experiments, calcium-dependent complement opsonization via the classical and mannose-binding lectin pathways was inhibited by adding magnesium and EGTA (final concentrations 10 mM and 8 mM, respectively) to 50% PHS in PBS without cations [14].

2.6. Measuring C3 and immunoglobulin deposition

To quantify C3, opsonized bacteria were incubated with FITC-conjugated goat anti-human-C3 antibodies (Cappel) for 60 min, washed, and assayed for fluorescence in a Novostar fluorometer (BMG Labtech, Offenberg, Germany) at equivalent concentrations. IgG and IgM opsonization were determined similarly using FITC-conjugated goat anti-human IgG and IgM antibodies (Cappel, MP Biomedicals, Irvine, CA). Fluorescence was normalized to the signal obtained from serum-treated bacteria incubated with normal goat FITC-conjugated antibody. For measurement of C3 deposition by immunoblotting, washed bacteria were boiled in sample buffer in reducing conditions and separated by 4–12% SDS-PAGE (Invitrogen, Carlsbad California). Proteins were transferred to polyvinylidene difluoride membranes (Perkin Elmer, Boston,