Microbes and Infection 15 (2013) 816–827

www.elsevier.com/locate/micinf

Original article

Generation of protection against *Francisella novicida* in mice depends on the pathogenicity protein PdpA, but not PdpC or PdpD

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Received 23 April 2013; accepted 15 July 2013
Available online 20 July 2013

Abstract

Previous results suggest that mutations in most genes in the *Francisella* pathogenicity island (FPI) attenuate the bacterium. Using a mouse model, here we determined the impact of mutations in *pdpA*, *pdpC*, and *pdpD* in *Francisella novicida* on *in vitro* replication in macrophages, and *in vivo* immunogenicity. In contrast to most FPI genes, deletion of *pdpC* (*Fn*Δ*pdpC*) and *pdpD* (*Fn*Δ*pdpD*) from *F. novicida* did not impact growth in mouse bone-marrow derived macrophages. Nonetheless, both *Fn*Δ*pdpC* and *Fn*Δ*pdpD* were highly attenuated when administered intradermally. Infected mice produced relatively normal anti-*F. novicida* serum antibodies. Further, splenocytes from infected mice controlled intramacrophage *Francisella* replication, indicating T cell priming, and mice immunized by infection with *Fn*Δ*pdpC* or *Fn*Δ*pdpD* survived secondary lethal parenteral challenge with either *F. novicida* or *Francisella tularensis* LVS. In contrast, deletion of *pdpA* (*Fn*Δ*pdpA*) ablated growth in macrophages *in vitro*. *Fn*Δ*pdpA* disseminated and replicated poorly in infected mice, accompanied by development of some anti-*F. novicida* serum antibodies. However, primed Th1 cells were not detected, and vaccinated mice did not survive even low dose challenge with either *F. novicida* or LVS. Taken together, these results suggest that successful priming of Th1 cells, and protection against lethal challenge, depends on expression of PdpA.
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Keywords: *Francisella*; Pathogenicity island; T lymphocytes; Protective immunity; Mice

1. Introduction

Francisella tularensis is a gram negative intracellular bacterium that replicates in the cytoplasm of infected eukaryotic cells, especially macrophages, and is the causative agent of the human disease known as tularemia [1,2]. Two subspecies of *F. tularensis*, *F. tularensis* spp. tularensis (also known as Type A) and *F. tularensis* spp. holarctica (Type B), are responsible for most human infections [3]. The resulting disease ranges from an acute course with a high potential mortality rate to milder but protracted systemic illness. A live attenuated vaccine strain, denoted LVS, was derived empirically from Type B *Francisella* by repeated passage, and appears to provide at least partial protection [4]; however, LVS has not been systematically studied for efficacy in human clinical trials, and is not currently licensed in the U.S. [2,5]. LVS is virulent for mice when administered by some routes, and has been used extensively as an experimental model of intracellular bacterial infections in general and *Francisella* in particular.

Human infections with another closely related strain, *Francisella novicida*, have been recognized only rarely, and

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1286-4579/$ - see front matter Published by Elsevier Masson SAS on behalf of Institut Pasteur.
http://dx.doi.org/10.1016/j.micinf.2013.07.005

thus *F. novicida* is not generally considered a human pathogen [6,7]. In contrast to humans, *F. novicida* is quite virulent for mice by most routes of infection [8–10]. *F. novicida* has proven to be much more amenable to genetic manipulation than other species of *Francisella*, and thus has been frequently invoked for studies of bacteria with defined mutations [11].

The discovery of a genetic element with hallmark features of a pathogenicity island in *Francisella* [12] immediately prompted interest in the contributions to virulence of the genes in this 30 kB region. The *Francisella* pathogenicity island (FPI) has been found in all *Francisella* species and strains examined to date, and is duplicated in Type A *Francisella*, Type B *Francisella*, and LVS. However, *F. novicida* contains only one copy of the FPI, further increasing its appeal for genetic studies. Previous studies examined defined mutants of *F. novicida* in each of the 18 FPI genes, and clearly demonstrated that deletion of 14 of the 18 genes greatly reduced bacterial replication in the J774 mouse macrophage cell line [13]. Thus, *pdpA*, *pdpB*, *dotU*, *vgrG* and *iglABCDEFGHIJ* were required for intracellular growth, but *pdpCDE* and *anmK* were not. Similarly, using transposon-derived mutants, replication of *F. novicida* in a mosquito cell line was dependent on *pdpA*, *pdpB*, and *iglABCD*, but not *pdpC* or *pdpD* [14]. Detailed studies of *F. novicida* Δ*pdpA* (*Fn*Δ*pdpA*) determined that this mutant does not escape from phagosomes of infected macrophages as does wild type *F. novicida*, and the gene produces a soluble protein [15,16]. However, the specific function of the gene and its protein remains unknown, as is also the case for the outer membrane protein PdpD, which affects virulence but not intramacrophage replication [17].

Some, but not all, of these mutants have begun to be characterized during *in vivo* mammalian infection. Mutations in *pdpA* not only reduced intramacrophage growth, but also attenuated intradermal (i.d.) infections of inbred mice: while the i.d. LD_{50} of wild type *F. novicida* in BALB/cByJ male mice is $\sim 2-5 \times 10^2$ [8], mice infected with up to 10^7 *F. novicida* Δ*pdpA* (*Fn*Δ*pdpA*) did not die [12,15].

Because *F. tularensis* has been considered a potential bioterrorism agent, recently there has been heightened interest in advancing licensure of LVS, as well as exploring next-generation vaccine candidates [18]. Proposed options include live attenuated *Francisella* strains, including *F. novicida*, with defined mutations, as well as various static subunit preparations. Given this interest as well as ongoing interest in pathogenic mechanisms in *Francisella*, here we performed detailed characterization of the impact of mutations in *pdpA*, *pdpC*, and *pdpD* on *F. novicida* infections of mice. Further, we determined the effect of these deletions on murine immunogenicity in terms of systemic anti-*Francisella* antibody production, T cell functions, and subsequent secondary lethal challenge.

2. Materials and methods

2.1. Experimental animals

Wild type BALB/cByJ and C57BL/6J control male and female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a facility at the Center for Biologics Evaluation and Research, fed autoclaved food and water *ad libitum*, and used between eight and twelve weeks of age. All experiments were performed under protocols approved by the CBER/FDA.

2.2. Bacteria, growth conditions, and preparation of infection stocks

F. tularensis LVS (ATCC 29684; American Type Culture Collection, Rockville, MD) and *F. novicida* strain U112 (originally obtained from Dr. Francis Nano, University of Victoria, British Columbia, CA) were cultured on modified Mueller-Hinton (MH) agar plates or in modified MH broth (Difco Laboratories, Detroit, MI) supplemented with ferric pyrophosphate and IsoVitalex (Becton Dickinson, Cockeysville, MD) as described previously [19]. Aliquots of bacteria were frozen in broth alone at −70 °C and thawed for individual experiments. Viable bacteria were quantified by plating serial dilutions on MH agar plates that were incubated for 1 day (*F. novicida*) or 3 days (LVS) at 37 °C in 5% CO_2. All stocks were confirmed as "fully virulent" by determining that the i.p. LD_{50} in BALB/cByJ male mice was <3 CFU [20].

The construction and bacteriological characterization of *F. novicida* strains, based on *F. novicida* U112, with defined deletions in the genes for *pdpA* [15] and *pdpD* [17], and associated complemented strains, have been previously described. The Δ*pdpC* deletion mutant was constructed essentially as previously described for *Fn*Δ*pdpA* [15]. Briefly, the region upstream of *pdpC* was PCR amplified using primers TAAGGGTAAGAGGAGATTTATATGAGTCAG and CATATTTTTGCATCCTTAAGCTAATGTACTTCCTTAATTTATC. The region downstream of *pdpC* was amplified with primers GATAAATTAAGGAAGTACATTAGCTTAAGGATGCAAA AATATG and TCTCAAGGAAGCTTGCCAGT. The two amplicons were designed to have overlapping regions, and these were used to fuse the upstream to the downstream region using overlap PCR [21]. The fused amplicon was further amplified using primers CTCGAGCTTAGTCACTATGGATGC and CTCGAGAGGTTAAGCACCGCAAGCTA. The final PCR product was ligated to a selection/counterselection cassette, and transformed into *F. novicida*. Integration and excision events led to the generation of a strain deleted in *pdpC*.

2.3. F. novicida *and* F. tularensis *LVS parenteral infections*

Mice were infected with the indicated bacteria intradermally (i.d.) by administering the indicated numbers of bacteria in 100 μl at the base of the tail, or intraperitoneally (i.p.) in 500 μl, diluted in sterile phosphate buffered saline (PBS) (Lonza, Walkersville, MD) containing <0.01 ng/ml of endotoxin. Actual bacterial doses were determined by simultaneous plate count. Blood samples were obtained at the indicated time points, sera prepared and frozen, and sample assessed for levels of anti-*Francisella* antibodies as described below.