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Francisella tularensis: Taxonomy, Genetics, and Immunopathogenesis of a Potential Agent of Biowarfare

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Abstract

Tularemia is a zoonosis of humans caused by infection with the facultative intracellular bacterium *Francisella tularensis*. Interest in *F. tularensis* has increased markedly in the past few years because of its potential use as an agent of bioterrorism. Five subspecies of this organism are found in the Northern hemisphere, but only *F. tularensis* subsp. *tularensis* and subsp. *holarctica* cause disease in humans. This review summarizes what is known about the pathogenesis of tularemia with a focus on bacterial surface components such as lipopolysaccharide and capsule as well as information obtained from the *F. tularensis* subsp. *tularensis* SCHU S4 genome. In particular, the mechanisms of action of recently identified virulence factors are discussed in the context of bacterial replication in macrophages and manipulation of the host inflammatory response. Throughout this report shared and unique features of *F. tularensis* subsp. *tularensis*, subsp. *holarctica*, and subsp. *novicida* are discussed.

Keywords

lipopolysaccharide; genome; virulence factor; macrophage; cytokines

INTRODUCTION

The anthrax attacks that occurred during the fall of 2001 dramatically alerted Americans to the potential deadly effects of bioterrorism and prompted the government of the United States to support research into the development of diagnostic, preventive, and therapeutic measures to combat organisms with biowarfare potential. The use of biological agents as weapons has a long history that predates our understanding of the microbial basis of infectious disease (12, 29,64,80,114). As early as 400 B.C., Scythian archers dipped their arrowheads in feces or decaying cadavers, and during the Mongol siege of Kaffa in 1347 plague-infected bodies were hurled into the city by the Tartars. In 1650, a Polish artillery officer suggested that projectiles filled with the saliva of rabid dogs could be used to cause epidemics, and during the American Revolutionary War blankets contaminated with variola were given to Native Americans. Before World War II, Japan, France, Germany, the Soviet Union, and Great Britain all had active biological weapons programs and the United States began similar activities in the early 1940s.

Pursuant to an executive order, the United States terminated its biological weapons program in 1970 and all stockpiles of infectious agents were destroyed by 1973 (29). Consequently, there was hope that this form of warfare would be abandoned worldwide. Indeed, the Biological Weapons Convention was implemented in 1972 and biowarfare was renounced by 140 nations (29). Nevertheless, the number of countries or other entities that possess toxic biological agents is increasing (80). This finding, together with the potential severity of natural infections,

necessitates an understanding of mechanisms of pathogenesis and the human immune response in order to develop appropriate treatments, preventive vaccines, and other defensive measures.

TAXONOMY AND GEOGRAPHIC DISTRIBUTION

In 1911, an outbreak of plague-like illness in ground squirrels in Tulare County, California, led to the discovery of a new pathogen by McCoy & Chapin (88). Shortly thereafter, it was appreciated that these hardy, nonspore-forming bacteria could persist in the environment for long periods and cause disease in humans. In honor of Edward Francis, these small, nonmotile, gram-negative cocco-bacilli are now called *Francisella tularensis.*

Francisella is the only genus within the family *Francisellaceae* and, on the basis of small subunit RNA sequences, is a member of the γ-subclass of proteobacteria (49). The organism most closely related to *Francisella* is *Wolbachia persica*, a tick endosymbiont (49). As defined by DNA similarity and fatty acid composition, there are two species within the *Francisella* genus: *tularensis* and *philomiragia* (49,67). In addition, there are five subspecies of *F. tularensis: tularensis* (also called *F. tularensis* type A), *novicida*, *mediasiatica*, *holarctica* (*F. tularensis* type B), and a variant of *holarctica* found in Japan (113) (Figure 1). Of these, only *F. tularensis* subsp. *tularensis* and subsp. *holarctica* cause disease in humans; and highly virulent type A organisms are evolutionarily older than moderately virulent type B bacteria (113). The live vaccine strain (LVS) is an attenuated variant of *F. tularensis* subsp. *holarctica* and *F. philomiragia* is a muskrat pathogen.

F. tularensis is widely distributed in the Northern hemisphere and is capable of infecting hundreds of different vertebrates and invertebrates (97). Recent studies have shown that *F. tularensis* is resistant to killing by the free-living amoebae, *Acanthamoeba castellanii* (1). In Norway, environmental infection of *Acanthamoeba* with *F. tularensis* may be a major source of outbreaks of gastrointestinal and respiratory tularemia in humans (16). The most important mammalian species involved in human infection include lagomorphs, voles, mice, squirrels, muskrats, and beavers. Infectious transfer to humans and other mammals can occur through aerosolization of infected material, the bite of an insect (ticks), and contact with infected animal products. *F. tularensis* is present throughout the continental United States, and infections caused by this organism were common prior to World War II but have declined dramatically since the 1950s (97). The current case rate is approximately 0.05 reported infections per 100,000 population, with 60% of all cases occurring in Midwestern states, such as Arkansas, Missouri, South Dakota, and Oklahoma (23a).

All *F. tularensis* subspecies and other *Francisellaceae* can be differentiated biochemically (97) and genetically by polymerase chain reaction (PCR) amplification of regions of difference (21). Recent studies have further divided *F. tularensis* subsp. *tularensis* into two genetically distinct clades, type A_1 and type A_2 (47). *F. tularensis* subsp. *tularensis* and subsp. *holarctica* also differ in geographic distribution (47). Type A_1 organisms are found predominately in the Midwest, California, and Massachusetts, and type A_2 bacteria have been detected in California and in the Mountain states. In marked contrast, *F. tularensis* subsp. *holarctica* is less common in North America and is distributed across Europe and Asia.

POTENTIAL FOR USE IN BIOWARFARE

F. tularensis subsp. *tularensis* is considered a potential bioweapon because of its extreme virulence, low infectious dose, ease of aerosol dissemination, and capacity to cause severe illness and death. Indeed, inhalation of as few as 10 colony-forming units (CFU) is sufficient to cause disease in humans and 30% to 60% of untreated infections can be fatal (109,110). The highly infectious nature of *F. tularensis* has been appreciated since the mid-twentieth century owing to water-borne outbreaks in Europe and the Soviet Union, laboratory accidents, and

epizootic cases in the United States (52,69,70,115). The largest naturally occurring outbreak of airborne tularemia occurred in Sweden in 1966–1967, during which more than 600 farmers were infected with *F. tularensis* type B (41).

Japanese germ warfare research units studied *F. tularensis* subsp. *tularensis*, and this organism may have been used against Chinese civilians, Russian troops, and American prisoners of war between 1932 and 1945 (65). In addition, the appearance of tularemia in thousands of Russian and German troops at the siege of Stalingrad may have been the result of deliberate use by the Soviets (3). However, a natural cause for this outbreak has not been eliminated, and military personnel may have acquired *F. tularensis* from mice and rats whose numbers multiplied owing to the widespread disruption of sanitation and hygiene during battle (39). During the Cold War both the Soviet Union and the United States prepared and stockpiled tons of infectious agents for potential use against the civilian populations of their enemies (29). In 1969, a World Health Organization expert committee estimated that aerosol dispersal of 50 kg of virulent *F. tularensis* bacteria over a metropolitan area with five million inhabitants would result in 250,000 casualties requiring extensive medical care and 19,000 deaths (42,104a).

BACTERIAL STRUCTURE

Lipopolysaccharide

Lipopolysaccharide (LPS, or endotoxin) is the major component of the outer membrane of gram-negative bacteria (103), and the interaction of LPS/MD-2 complexes with Toll-like receptor 4 (TLR4) on macrophages and endothelial cells activates a signaling cascade that results in the release of proinflammatory cytokines (23,56,103). *Escherichia coli* LPS consists of a hexaacylated glucosamine disaccharide phosphorylated at the 1- and 4′-positions (lipid A) that is modified further by the addition of core oligosaccharides and a variable number of additional sugars that form the O-antigen (103). Alterations in LPS structure, including changes in phosphorylation or fatty acid chain length, position, and number, significantly affect endotoxin bioactivity (103,116). In this regard, it is noteworthy that the LPS of *F. tularensis* is 1000-fold less potent than the LPS of enteric bacteria (4,106) and does not activate cells via TLR4 (106,117).

Recent studies have begun to define unique structural features of *Francisella* LPS that may account for its low bioactivity. The LPS of both *F. tularensis* subsp. *holarctica* and LVS is a tetraacylated structure that lacks fatty acids at the O-3 position of hexosamine II in the lipid A backbone (Figure 2), and compared with *E. coli*, the fatty acids that are present are unusually long (98,125). LVS lipid A is not phosphorylated at either the 1- or 4′-position (125). Dephosphorylation of the 1-position is catalyzed by LpxE and requires prior LPS transport across the inner membrane by ValA, a homolog of the ABC transporter MsbA (89,90,127). In contrast to LVS, *F. tularensis* subsp. *holarctica* LPS contains a galactosamine-1-phosphate linked to the reducing terminus of the lipid A disaccharide (Figure 2) (98). A homolog of the *Salmonella enterica* serovar *Typhimurium* arabinosamine transferase, ArnT, is present in the SCHU S4 genome and may encode a galactosamine transferase (98). Modification of *Salmonella* lipid A with arabinose confers resistance to polymyxin B and cationic antimicrobial peptides (103), but whether galactosamine has a similar effect on *Francisella* LPS is unclear.

Structural analysis indicates that the core region of *F. tularensis* LPS contains a single 3-deoxy-D-manno-octulosonic acid and lacks heptose (125). Specifically, the structure of the core region sugars is

$$\begin{array}{ll} \alpha- & \mathrm{Glc}-(1\rightarrow 3) \\ & \quad\vert \\ & \mathrm{R}-4)\mathrm{b}-\mathrm{Man}(1\rightarrow 4)-\alpha-\mathrm{Man}-(1\rightarrow 5) \\ & \quad\times \mathrm{Kdo}(2\rightarrow 6)-\beta-\mathrm{GlcN}-(1\rightarrow 6)-\mathrm{GlcN} \\ & \quad\vert \\ \alpha- & \mathrm{GalNAc}-(1\rightarrow 2)\beta-\mathrm{Glc}-(1\rightarrow 2) \end{array}$$

The O-antigens of *F. tularensis* subsp. *tularensis* and subsp. *holarctica* LPS are identical, but the O-antigen of *F. tularensis* subsp. *novicida* is distinct (101,119,124,126). The repeating subunits of the *F. tularensis* subsp. *tularensis* and subsp. *novicida* O-antigens are (124)

F. tularensis subsp. *tularensis*

$$\begin{array}{l} \rightarrow 4)-\alpha-\mathrm{D}-\mathrm{GalNAcAN}(1\rightarrow 4)- \\ \alpha-\mathrm{D}-\mathrm{GalNAcAN}-(1\rightarrow 3)-\beta- \\ \mathrm{D}-\mathrm{QuiNAc4Ac}-(1\rightarrow 2)- \\ \beta-\mathrm{D}-\mathrm{Qui4NFo}-(1\rightarrow . \end{array}$$

F. tularensis subsp. *novicida*

$$\begin{array}{l} \rightarrow 4)-\alpha-\mathrm{D}-\mathrm{GalNAcAN}(1\rightarrow 4)-\alpha- \\ \mathrm{D}-\mathrm{GalNAcAN}-(1\rightarrow 4)-\alpha-\mathrm{D}- \\ \mathrm{GalNAcAN}-1\rightarrow 3)-\alpha-\mathrm{D}- \\ \mathrm{QuiNAc4NAc}-(1\rightarrow . \end{array}$$

The O-antigen gene cluster in the SCHU S4 genome contains an insertion element (IS-Ftu1) that is flanked by transposase genes, and a similar insertion element in *Shigella sonnei* is essential for virulence (77,101). ISFtu1 may be responsible for *Francisella* LPS phase variation, which in turn modulates bacterial fate in murine macrophages (37,72).

Capsule

Wild strains of *F. tularensis* and LVS possess a surface exopolysaccharide capsule (68,105, 112), and our data demonstrate the presence of immunogenic surface capsular material on *F. tularensis* subsp. *holarctica* strain 1547 (Figure 3). It has been known for some time that the *F. tularensis* capsule protects bacteria from killing by serum complement (105,112) and a cluster of genes putatively involved in polysaccharide biosynthesis is present in the SCHU S4 genome (77). Nevertheless, a detailed analysis of the capsule has not been performed and our understanding of its composition is incomplete. In the 1970s biochemical studies found that capsular extracts of SCHU S4 contain carbohydrates (including mannose, rhamnose, and two unidentified dideoxy sugars), as well as amino acids and α-OH 14:0 and 16:0 fatty acids (68). A role for capsule in virulence is suggested by the finding that bacteria from which the capsular material has been extracted do not cause disease in mice or guinea pigs (68). Whether capsule synthesis is regulated in vivo or in vitro is unknown. However, continued growth on synthetic media can restore encapsulation and virulence to a relatively avirulent, unencapsulated strain of LVS (28), and recent data suggest that capsule may be downregulated by LVS inside macrophages (57).

Pilus

Orthologs of genes required for type IV pilus expression are present in the *F. tularensis* subsp. *tularensis* SCHU S4 genome (55,77), and microscopy data demonstrate the presence of structures that resemble type IV pili on the surface of LVS (55). In other organisms type IV pili mediate bacterial attachment to host cells, DNA uptake, twitching motility, and biofilm

formation (22,24,95). Nevertheless, the role of pili in *Francisella* physiology and virulence has not been determined.

GENOME

The genome of *F. tularensis* subsp. *tularensis* SCHU S4 is small, approximately 1.9 Mb, and contains 1804 predicted coding sequences, of which 302 are unique to *Francisella* (77). Five of the predicted coding sequences unique to strain SCHU S4 form a new protein family of unknown function (77). Because no obvious virulence factors were predicted by the genome, the relatively large number of *Francisella*-specific predicted coding sequences might provide the bacterium with novel uncharacterized factors responsible for the high virulence of this organism. Thus, it is of interest that *F. tularensis* subsp. *tularensis* does not have either of the plasmids found in LVS or *F. tularensis* subsp. *novicida* (77,96,100).

A large proportion of inactivated genes characterize the SCHU S4 genome. As a result of insertions, deletions, and substitution mutations, more than 10% of all genes are pseudogenes or gene fragments; and 14% of all pseudogenes can be accounted for by the presence of five insertion elements (IS-Ftu 1–5) scattered throughout the genome (77,120). The decaying genome of *F. tularensis* has disrupted more than half of the predicted metabolic pathways for which genes are present and explains the fastidious growth requirements of the organism (77). Fourteen compounds are essential to sustain growth of *Francisella* because of absent or incomplete enzymatic pathways.

A unique 33.9-kb region of DNA is duplicated in the SCHU S4 genome (77). The 25 genes encoded within this region have no known bacterial homologs and may encode as yet unidentified virulence determinants. However, the presence of this genomic island in LVS as well as *F. tularensis* subsp. *novicida* indicates that these genes alone cannot account for the high virulence of *F. tularensis* subsp. *tularensis* (91). Because disruption of genes within this region generates mutant bacteria that are attenuated for survival in macrophages, this region of the chromosome has been called the Francisella pathogenicity island (FPI) (58,61,75,91).

Compared with other bacteria, *F. tularensis* has relatively few transcriptional regulators (77). MglA is the only regulatory protein described to date. Encoding proteins homologous to the *E. coli* stationary-phase regulators SspA and SspB, the *mglAB* operon is required for *F. tularensis* subsp. *novicida* intracellular growth and intra-amoeba survival (14,78). MglA regulates expression of several FPI genes, including *iglC*, *iglA*, *pdpA*, and *pdpD* (78).

F. tularensis does not appear to secrete toxins, and genes encoding type III, IV, or V secretion systems have not been identified in the SCHU S4 genome. However, homologs of pilin and pseudopilin genes associated with type II secretion are present (55), along with 15 potential ATP-binding cassette systems that may be involved in type I secretion (77).

Although iron is an essential nutrient for *F. tularensis*, genes encoding TonB and receptors for siderophores, transferrin, lactoferrin, or other iron-containing compounds are not present in the SCHU S4 genome (50,77). Nevertheless, a gene encoding the ferric uptake protein, Fur, and genes regulated by Fur (FTT0030) are present, indicating that expression of some genes may be iron dependent (77).

Genetic Tools for Manipulation of *Francisella tularensis*

Efforts to understand *F. tularensis* pathogenesis have been hindered by of a lack of reliable methods for genetic manipulation of the virulent *Francisella* subspecies and by the inability to make targeted mutations. Because *F. tularensis* subsp. *novicida* is more genetically amenable by standard transformation techniques, most genetic manipulation has been

conducted in this bacterium. However, recent efforts of several researchers have provided the *Francisella* community with tools to introduce DNA into the other subspecies of *Francisella* and to make specific mutations. These methods have been successfully employed in A- and B-type strains, creating mutations in many genes including those encoded within the FPI.

pFNL10, a cryptic plasmid isolated from *F. tularensis* subsp. *novicida* strain F6168, is the basis for all *F. tularensis*–replicating plasmids (96). The origin of *Francisella* replication in this plasmid was determined by deletion and sequence analysis (96,100). Using pFNL10, *E. coli*–*F. tularensis* shuttle vectors were created, but these plasmids are not stably maintained within *F. tularensis* (73,86,94). A stable *Francisella* plasmid, pFNLTP1, was created by spontaneous deletion during passage through LVS (86). A conditionally replicating derivative of pFNLTP1, pFNLTP9, was created as a vector for allelic exchange or transposon delivery (86).

Conjugation, transformation, electroporation, and cryotransformation have been used to introduce DNA into *F. tularensis*. Conjugation has been performed in both the SCHU S4 strain and LVS to create mutations (60,122). In LVS, transfer is most efficient within 18 h of mating and at 25°C (60). Chemical transformation of DNA has been used successfully only in *F. tularensis* subsp. *novicida*, and efficiency of DNA uptake depends on the plasmid construct and the length and source of DNA Moreover, *Francisella* DNA is transformed with higher efficiency than non-*Francisella* DNA (10,123). Attempts to transform LVS using this method were unsuccessful (10). Electroporation is an efficient method of introducing DNA into type A *Francisella* spp., LVS, and *F. tularensis* subsp. *novicida* (10,86,100). Transformation efficiency of electroporated DNA is approximately $3–6 \times 10^7$ CFU g^{-1} of DNA in LVS and 2×10^3 CFU g^{-1} of DNA in *F. tularensis* subsp. *novicida* (86). Transformation efficiency of *F. tularensis* subsp. *novicida* is 2×10^8 when *novicida* DNA is transformed, suggesting the presence of a restriction modification system; no such system has been observed in LVS (86). Subspecies and strains of *Francisella* transform with different efficiencies and the presence of an exopolysaccharide capsule may affect DNA uptake (10,13,86,100). Cryotransformation is an efficient and reliable method of introducing DNA into *F. tularensis* subsp. *novicida* and subsp. *holarctica*, and it is a fast procedure because competent cells do not need to be made (75,96).

Mutational analysis is critical to elucidating the pathogenesis of *F. tularensis. Francisella* mutants were initially created using random transposon mutagenesis of *F. tularensis* DNA in *E. coli*, followed by transformation into *F. tularensis* and allelic exchange (10,14,17,36,61). More recently, stable random mutants have been constructed in LVS using the Epicentre EZ::TN transposon mutagenesis system (71). Allelic replacement using the suicide plasmid pPV was used to mutate *iglC* in LVS and *FTT0918* in strain SCHU S4; both genes are required for intracellular survival (60,122).

IMMUNOPATHOGENESIS

Infection of Macrophages

F. tularensis can infect macrophages of humans, mice, rats, rabbits, and guinea pigs, and it is generally believed that macrophages are the major reservoir of *Francisella* in vivo. In the past few years several research groups have begun to define the niche occupied by this organism inside host cells and to delineate how bacterial growth and survival are modulated by specific virulence factors.

Receptors and phagocytosis—Internalization of microbes and particles via phagocytosis requires specific receptor-ligand interactions and is driven by local actin polymerization (2). The binding interactions that confer uptake of *Francisella* by phagocytes are only beginning to be defined. Of note in this regard is the fact that cells of the commonly used murine

macrophage cell line J774 are remarkably resistant to *F. tularensis* (in standard tissue culture media), and high multiplicities of infection (MOI of 100:1 to 500:1) are needed achieve uptake of one or two bacteria per cell (74,76,117). Phagocytosis of *F. tularensis* by primary murine bone marrow–derived macrophages and human monocytes is also inefficient (18,31). These data contrast sharply with the notion that macrophages are the major intracellular reservoir of *F. tularensis* in vivo and the fact that as few as 10 organisms can **c**ause severe disease. Therefore, in vitro model systems may not be optimized. Indeed, recent data indicate that infection of human monocyte-derived macrophages (MDM) or monocyte-like THP-1 cells by a clinical *F. tularensis* isolate or LVS is enhanced markedly by fresh serum containing active complement factors (30,31). Under these conditions uptake of opsonized bacteria is mediated in part by complement receptor 3 (CD11b/CD18) and occurs via the extension of large asymmetric pseudopod loops that project from the macrophage surface to encircle attached bacteria (31).

Taken together, the data suggest that serum opsonins are required for optimal uptake of F. tularensis by mononuclear phagocytes. Nevertheless, in future studies it will be important to compare directly the ability of F. tularensis subspecies to infect different types of mononuclear phagocytes including alveolar macrophages in the presence and absence of opsonins and to assess whether infection efficiency is modulated by surface virulence factors such as capsule or LPS. In this regard it is of interest that both capsule-deficient organisms and LPS-phase variants are serum sensitive (36,66,105,112). Moreover, diminished O-antigen content impairs virulence in mice and reduces survival in macrophages in vitro (36,66).

Phagosome escape and replication in the cytosol—Under normal circumstances nascent phagosomes undergo a maturation process during which the composition of this compartment is modified by sequential interactions with early endosomes, late endosomes, and lysosomes (2). In the mature phagolysosome, oxidants, cationic antimicrobial peptides, and lysosomal acid hydrolases act in concert to kill and degrade ingested bacteria. Pathogens that replicate inside macrophages must either evade or withstand this hostile microenvironment. It has been known for some time that LVS phagosomes do not fuse with thorium-loaded lysosomes in J774 cells (7) and that ingested bacteria begin to replicate after a lag of approximately 6 h (7,50). The composition of the *Francisella* phagosome was not determined in these early studies. However, phagosome neutralization inhibits LVS replication, which suggested that *F. tularensis* might reside in an acidic vacuole that allows access to iron and other essential nutrients (50). By contrast, recent ultrastructural analyses demonstrate that both clinical *F. tularensis* isolates and LVS escape the phagosome in primary human and murine macrophages (as well as transformed macrophage cell lines) and replicate in the cytosol (30,57). Confocal microscopy studies have begun to define the nature of the compartment from which *F. tularensis* escapes. Nascent phagosomes containing live clinical *F. tularensis* isolates, LVS, or *F. tularensis* subsp. *novicida* associate transiently with early endosomes and thereafter accumulate the late-endosome markers lamp-1, lamp-2, and CD63 (30,57,107). However, further maturation of the *F. tularensis* phagosome does not occur; these compartments are only moderately acidified (pH 6.7) and do not acquire lysosomal hydrolases (30,107). A progressive decline in phagosome lamp-1 and CD63 beginning 3 to 4 h into infection correlates directly with disruption of the phagosome membrane and with the presence of bacteria in the cytosol (30,57).

That all *F. tularensis* isolates studied to date escape the phagosome and replicate in the cytosol of human and murine macrophages suggests that this process is controlled by conserved virulence determinants. Nevertheless, how the phagosome membrane is breached is unknown. Virulence factors with homology to listerolysin O have not been described in *F. tularensis*, and synthesis of relatively few proteins is affected during intracellular infection (58). Transposon mutagenesis of *F. tularensis* subsp. *novicida* demonstrated that *iglB* and *iglC* are

essential for intramacrophage growth (61). IglC is a 23-kDa protein that is upregulated inside macrophages (58), and the intracellular growth locus, *iglABCD*, defines an operon that is a major component of the FPI (91). Expression of genes in the FPI is controlled by the transcriptional regulators MglA and MglB (macrophage growth locus A and B) (78). Disruption of *mglA*, *mglB*, *iglB*, or *iglC* inhibits phagosome escape, intramacrophage growth, and virulence in mice (14,61,75,78,108). Other FPI genes controlled by MglA include *pdpA*, *pdpB*, *pdpC*, and *pdpD* (78,91). The function of the Pdp proteins is unknown; however, it is of interest that *pdpD* is present in type A organisms, altered in *F. tularensis* subsp. *novicida*, and absent in type B strains including LVS (91).

Additional proteins required for intracellular survival are alanine racemase, glutamine phosphoribosylpyrophosphate amido-transferase, ClpB heat-shock protease, and a 58-kDa protein of unknown function (61,122). Finally, the role of capsule and LPS in intramacrophage survival has not been defined precisely. Strains with rough LPS survive poorly in mice and macrophages (36,37,66), but whether phagosome maturation is affected directly has not been determined. The reduced fitness of these organisms may be indirect and may reflect membrane perturbations introduced by the membrane attack complex of the complement cascade.

Apoptosis—Replication of LVS in J774 cells culminates in cell death after approximately 24 h (7,58). Cytotoxicity requires bacterial replication and does not occur if phagocytosis is blocked or if ingested organisms are killed with ciprofloxacin (74). Dying cells exhibit features of apoptosis including DNA laddering and surface exposure of annexin V (74,75). Moreover, apoptosis is triggered by bacteria that reach the cytosol, as judged by the sustained viability of J774 cells infected with Δ*iglC* LVS or the phenotype of thioglycolate-elicited peritoneal macrophages that ingested *F. tularensis* subsp. *novicida* mutants Δ*mglA* or Δ*pdpA* (75,87).

Pathogen-induced macrophage apoptosis can favor either the host or the microbe (54). With regard to *Francisella*, two distinct mechanisms of apoptosis have been described (76,87). LVS infection of J774 cells (MOI 500:1) activates the intrinsic apoptosis pathway. Mitochondrial cytochrome *c* release, activation of caspases -9 and -3, and cleavage of poly-ADP ribose polymerase peak at ~18 h and viable bacteria are released from dying cells. Conversely, infection of thioglycolate-elicited peritoneal macrophages with opsonized *F. tularensis* subsp. *novicida* (MOI 30:1) triggers rapid apoptosis (90% death within 5 h) via the caspase-1 inflammasome pathway. This mechanism of cell death is an element of innate host defense, and deletion of caspase-1 markedly increases *F. tularensis* subsp. *novicida* virulence in mice during the first day post infection. The reasons for these divergent outcomes are unknown; differences in macrophage type and activation state, unique features of different *F. tularensis* subspecies, or triggering of distinct signaling pathways by opsonized and unopsonized bacteria could account for this discordance.

Role of Neutrophils

Neutrophils (also called polymorphonuclear leukocytes, or PMN) are understudied in the context of tularemia and whether they contribute to pathogenesis or host defense is controversial. The results of one study demonstrated that depletion of granulocytes with monoclonal antibodies markedly increases the lethality of LVS administered to mice via the dermis or the peritoneal cavity (111). Conversely, neutropenia does not exacerbate disease course when LVS is administered via the aerosol route (32). In this model, PMN depletion has no significant effect on bacterial burden in the lung and spleen, and the LVS load in the liver is only moderately elevated. Importantly, this host defense defect was not due to an absence of PMN in the alveoli of control animals, and as such the data suggest that neutrophils may not control *F. tularensis* in the microenvironment of the lung.

Relatively few studies have examined the interactions of *F. tularensis* with neutrophils isolated from the peripheral blood of humans or rhesus monkeys. Like most bacteria, binding of *F. tularensis* subsp. *holarctica* or LVS to neutrophils and their subsequent phagocytosis requires complement components in fresh serum (83,102). A central aspect of the killing arsenal of the neutrophil is the NAPDH oxidase, which is a multicomponent enzyme that catalyzes the conversion of molecular oxygen into superoxide anions (93). Superoxide anions rapidly dismutate to form hydrogen peroxide, and in the presence of myeloperoxidase released from primary granules, hydrogen peroxide is converted into highly toxic hypochlorous acid (HOCl) (92). Three studies performed more than 20 years ago examined whether a strain of *F. tularensis* subsp. *holarctica* or LVS activated neutrophils as judged by degranulation and synthesis of oxidants (83,84,102). The data indicate that *F. tularensis* opsonized with immune serum is a more potent stimulus than bacteria preincubated with normal human serum or specific antibodies alone. Moreover, normal serum does not support killing of *F. tularensis* by PMNs and >95% of ingested bacteria remain viable. In contrast, in the presence of immune serum approximately one third of virulent bacteria and nearly all attenuated organisms are eliminated. Furthermore, LVS is more susceptible than wild *F. tularensis* to superoxide and HOCl in a cell-free system (85). Taken together, the data suggest that oxidants contribute to *Francisella* control by neutrophils, that wild *F. tularensis* may be more resistant to reactive oxygen species than attenuated bacteria, and, most importantly, that oxidative mechanisms of host defense may not be effective during primary infection.

Virulence factors that modulate the phagocyte respiratory burst are largely undefined. A purified acid phosphatase (AcpA) inhibits the oxidative burst of porcine neutrophils stimulated with formyl peptides or phorbol esters (104). However, *acpA*-null mutants of *F. tularensis* subsp. *novicida* are not attenuated in mice (15). Thus, resistance to toxic oxidants may be multifactorial. In support of this notion, capsule-deficient variants of LVS are more resistant to killing by PMNs despite increased serum sensitivity (105), and inactivation of *minD* enhances *F. tularensis* subsp. *novicida* sensitivity to superoxide anions in vitro and impairs virulence in mice (8).

Cytokines and Phagocyte Activation in Host Defense

Interferon (IFN)-γ, tumor necrosis factor-α, and interleukin (IL)-12 play important roles in murine resistance to LVS and *F. tularensis* subsp. *novicida* (9,43,45,46,51,59,79,81). IL-12 is a potent stimulus for production of IFN-γ, which in turn induces nitric oxidase synthase (iNOS) and synthesis of NO by macrophages (11,38,51,62,63). NO is bacteriostatic (82) and studies of iNOS-null animals confirm a key role for reactive nitrogen intermediates in *Francisella* control (82). IFN-γ also limits bacterial growth inside murine alveolar macrophages by an NO-independent mechanism (99). In contrast to mouse cells, human macrophages generate NO under a limited set of conditions (121), and as such specific mechanisms of control relevant to human tularemia are not well defined. However, recent data indicate that IFN-γ enhances maturation of *F. tularensis* subsp. *novicida* phagosomes in human MDMs and impairs phagosome escape (107).

On the other hand, LVS disrupts secretion of proinflammatory cytokines by human mononuclear phagocytes (18,117,118) and human umbilical vein endothelial cells (48) and uncouples antigen presentation from cytokine production in murine pulmonary den-dritic cells (19). Determining the mechanism by which *Francisella* alters cytokine production is an area of active investigation. IglC impairs intracellular signaling pathways required for cytokine synthesis (117). In addition, the unusual structure of *F. tularensis* LPS likely accounts for its low bioactivity and may prevent cell activation via TLRs (106,117). Indeed, TLR4 has no significant role in host defense against *F. tularensis* subsp. *tularensis* in the lung or against LVS in the dermis (25,26). These data, together with the finding that *Francisella* LPS does not

act as an antagonist (4), suggest that the ability of *Francisella* LPS to interact with one or more of the host endotoxin-binding molecules that confer cell activation (LPS binding protein, CD14, or TLR4/MD-2) may be impaired. A role for LPS in modulating macrophage activation during the immune response is also of interest given the higher bioactivity of LPS in *F. tularensis* subsp. *novicida* organisms (72).

Late control and ultimate resolution of *F. tularensis* infection require T-lymphocytes (34,35, 46,128). Both $CD4^+$ and $CD8^+$ cells are required to combat SCHU S4, and the ability of *F. tularensis* subsp. *tularensis* to cause thymic atrophy and thymocyte depletion may account in part for the marked virulence of this *F. tularensis* subspecies (27,53). Conversely, the role of B-lymphocytes in primary tularemia is less clear. In humans antibody levels do not correlate with protection against infection (115) and B-cell-deficient mice do not succumb to sublethal doses of LVS (44). Nevertheless, B-cell-derived cytokines appear to modulate neutrophil influx and killing of infected hepatocytes by mechanisms independent of antibody production (33, 40), and as noted above, specific antibodies may be important as opsonins that favor killing of *F. tularensis* by neutrophils in the immune host. In support of this notion, B-cell-deficient mice exhibit profound neutrophilia and die secondary to PMN degranulation, tissue damage, and shock in response to a second dose of LVS (20).

CONCLUSIONS

Our understanding of *Francisella* pathogenesis is advancing rapidly. Sequencing of the genome and the development of tools for genetic manipulation of the organism have enabled researchers to identify novel virulence factors that impair macrophage function and disrupt the host immune response. Other unique features of *F. tularensis* were identified by structural analysis of LPS and other surface determinants. The wealth of information obtained in the past few years is substantial and the fast pace of new discoveries in this field will likely continue for some time.

Despite recent advances, many important questions have not yet been answered. For example, the features of *F. tularensis* subsp. *tularensis* and the aspects of the lung microenvironment that account for the ability of as few as 10 organisms to cause severe pneumonic tularemia in humans have not been defined. Also lacking is a clear understanding of the fate of *F. tularensis* in cell types other than macrophages. The extent to which *F. tularensis* interacts with and alters the function of endothelial cells, epithelial cells, neutrophils, and dendritic cells is largely unknown. Given the ability of *F. tularensis* to cause a wide spectrum of diseases, it is tempting to speculate that virulence factor expression may be affected by route of infection or bacterial residence in different tissues including the lung, skin, spleen, and liver. It is likely that answers to these questions (and many others) will be obtained in the near future and that the insights gained will advance our understanding of the pathogenesis of tularemia and provide information essential for the development of new vaccines.

SUMMARY POINTS

1. *Francisella* subspecies differ in geographic distribution and only *F. tularensis* subsp. *holarctica* and subsp. *tularensis* cause disease in humans.
2. The *F. tularensis* subsp. *tularensis* SCHU S4 genome is notable for its small size, the presence of a pathogenicity island, and a large number of pseudogenes that disrupt metabolic pathways.
3. *Francisella tularensis* subsp. *holarctica* LPS has an unusual structure and exhibits low bioactivity.

4. Opsonized *Francisella* enter macrophages via complement receptor 3 and escape the phagosome, and bacterial replication in the cytosol culminates in macrophage apoptosis.
5. LPS, surface capsule, and genes in the pathogenicity island are required for virulence in mice and replication in human and murine macrophages in vitro.
6. Resolution of infection requires T-cells and associated cytokines. B-cells and antibodies may modulate neutrophil function in the immune host.
7. Unique features of *Francisella* that account for the pronounced virulence of *F. tularensis* subsp. *tularensis* in humans, particularly in the context of pulmonary tularemia, remain obscure.

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Glossary

Pathogen

a microorganism that causes disease

Live vaccine strain (LVS)

an attenuated strain of *F. tularensis* subsp. holarctica previously used as a live vaccine

CFU

colony-forming unit

Lipopolysaccharide (LPS, or endotoxin)

the major surface component of gram-negative bacteria

TLR

Toll-like receptor

SCHU S4

genome strain of *F. tularensis* subsp. *tularensis*

D-GalNacAN

2-acetamido-2-deoxy-2-galacturonamide

Capsule
: a sugar-rich structure on the surface of some bacteria that can inhibit phagocytosis or prevent killing by serum complement

Virulence factor
: a component of a microorganism that is important for its ability to damage a host and cause disease

Francisella pathogenicity island (FPI)
: a distinct region of the genome containing genes that encode virulence factors

Igl
: intracellular growth locus

Mgl
: macrophage growth locus

Phagocytosis
: a specialized form of endocytosis that allows macrophages and neutrophils to engulf microorganisms

MOI
: multiplicity of infection

MDM
: human monocyte-derived macrophage

Opsonize
: binding of opsonins (serum complement factors and/or antibodies) to the surface of microbes to facilitate phagocytosis

PMN
: polymorphonuclear leukocyte, neutrophil

iNOS
: inducible nitric oxide synthase

Figure 1.
Evolutionary schema based on loss of regions of difference and extensive nucleotide variation. Adapted from Svennson et al. (113).

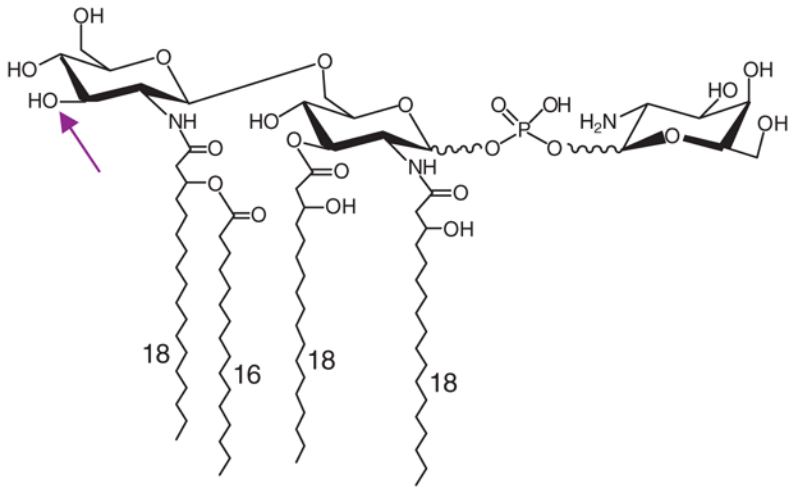

Figure 2.
The lipid A of *F. tularensis* subsp. *holarctica* strain 1547. Note the asymmetrical lipid A structure and the absence of O-acylation on hexosamine II (*arrow*). The phosphogalactosamine substitution on the 4′-position of this hexosamine is also unique to *Francisella.*

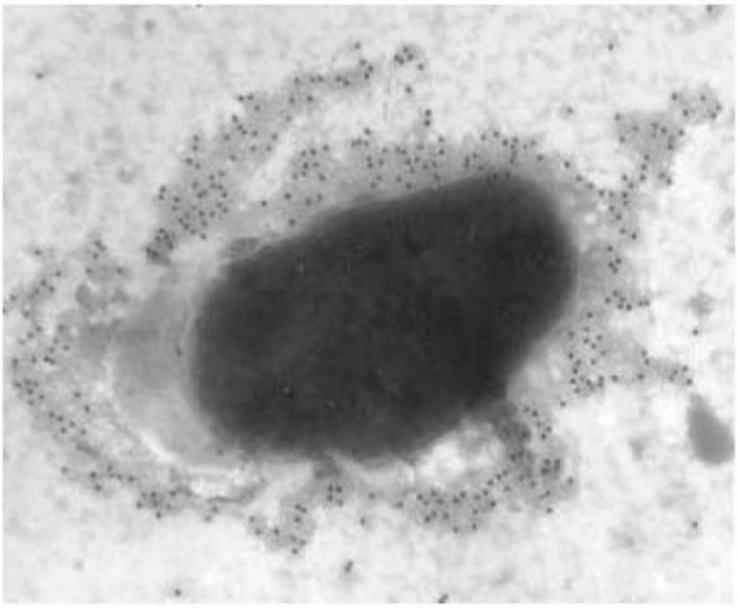

Figure 3.
A whole mount electron micrograph of *F. tularensis* subsp. *holarctica* strain 1547 fixed in glutaraldehyde with ruthenium red and stained with monoclonal antibody XE8 and secondary antibodies conjugated to colloidal gold to show the putative surface capsule. Magnification: ×15,000.