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Tularemia Pathogenesis and Immunity

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1. INTRODUCTION

Francisella tularensis, the etiological agent of tularemia, is one of the most infectious pathogens known. Human cases of the disease occur infrequently in the northern hemisphere, mainly in some parts of Scandanavia and in Russia.[1] It is probably the high infectivity, ease of culture, and low levels of natural immunity to the bacterium that originally attracted interest in *F. tularensis* as a bioweapon.[2] During the 1930s and 1940s the bacterium was evaluated by Japanese germ warfare units. Later, both the former Soviet Union (fSU) and the USA reportedly produced weapons capable of disseminating the bacterium.[2] The programme to develop biological weapons in the USA was abandoned in 1969. In other countries the status of the programme is not clear, and there are some suggestions that strains which are resistant to commonly available antibiotics have been developed for use as bioweapons.[2]

The severity of disease caused by *F. tularensis* is highly dependent on the causative strain and the route of entry of the bacterium into the host. Currently, there are four accepted subspecies (Table I), and *F. tularensis* subsp. *tularensis* is the most virulent in humans. Most naturally acquired cases of disease in humans are the consequence of a bite from an arthropod vector that has previously fed on an infected animal.[1] Ulceroglandular tularemia is the usual form of disease

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TABLE I
Some Properties of the Four Subspecies of *F. tularensis*

Subspecies	50% Lethal dose in humans	Identification and diagnostic tests		
		Citrulline ureidase	Glycerol fermentation	Glucose fermentation
Tularensis	10–50 cfu[3]	+	+	+
Holarctica	<10[3] cfu[3]	−	−	+
Mediaasiatica	NR	+	+	−
Novicida	>10[3] cfu[3]	NR	NR	+

cfu: colony forming units.

that develops, and is severely debilitating, but not often fatal.[1,2,4] Ticks such as *Dermacentor reticulatus* and *Ixodes ricinus* are the most frequent vectors, and mammals such as ground squirrels, rabbits, hares, voles, water rats and other rodents are believed to be the usual reservoirs of infection.

The inhalation of *F. tularensis* can result in the development of pneumonic disease. Naturally occurring cases of primary pneumonic tularemia are infrequent, and are usually a consequence of the inhalation of dusts from hay contaminated from infected rodents.[5–7] Most of the information on pneumonic tularemia comes from the infrequent cases that occur naturally and from trials with human volunteers in the USA during the 1950s.[8,9] Two naturally occurring outbreaks have attracted particular attention. Firstly, a number of cases of pneumonic tularemia were reported in Sweden during 1966–1967.[7] The disease was contracted by those working in farming communities, and the available evidence indicates that the bacteria were inhaled in dusts generated when contaminated hay was moved from storage sites in fields into barns. Secondly, there have been a number of cases of pneumonic tularemia on Martha's Vineyard in the USA.[10] The etiology of these cases is somewhat unusual, being associated with lawn mowing or brush cutting activities that resulted in the generation of airborne bacteria from the remains of rabbits that had died from tularemia.[10]

F. tularensis used as a bioweapon would be expected to be delivered by the aerosol route, and would most likely cause pneumonic tularemia.[2] Previous human volunteer studies in the USA have shown that the infectious dose of *F. tularensis* subsp. *tularensis* by the airborne route is between 10 and 50 cfu.[8] The World Health Organization has used this information to predict the number of casualties following a bioweapon attack with *F. tularensis* (Fig. 1).[11] Providing appropriate medical care for the large number of incapacitated casualties would pose significant logistical problems. Based on these predictions, the centers for disease control and prevention estimated that the cost to society of an airborne exposure to *F. tularensis* would be $5.4 billion for every 100,000 persons exposed.[2]

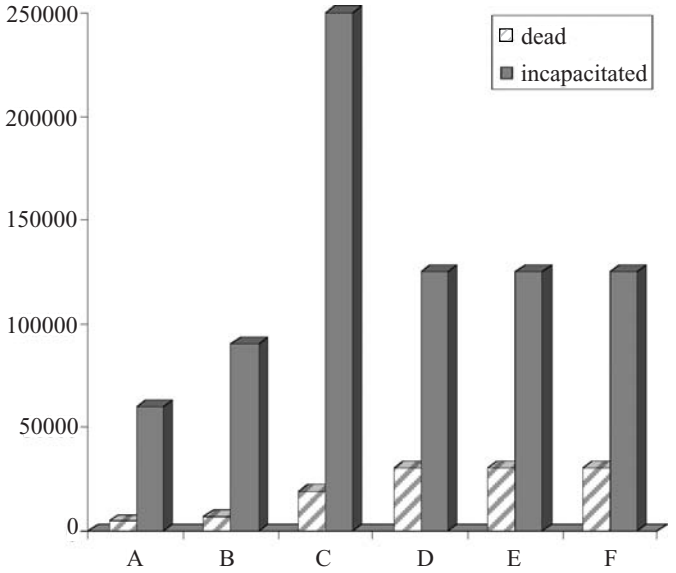

FIGURE 1. Number of deaths and number of cases of incapacitating disease following an airborne attack with 50 kg of dried *F. tularensis*. Exposure of an urban population of 500,000 (A), 1,000,000 (B) or 5,000,000 (C) in an economically developed country. Exposure of an urban population of 500,000 (D), 1,000,000 (E) or 5,000,000 (F) in a developing country. Data taken from *Health Aspects of Chemical and Biological Weapons,* 1970, World Health Organisation, Geneva.[11]

2. PATHOGENESIS

2.1. Human Disease

F. tularensis is able to enter the host after vector-borne delivery (i.e., across the skin) or by crossing a mucosal surface.[1] In the case of vector-borne delivery the most likely outcome is ulceroglandular tularemia. The typical incubation period for ulceroglandular tularemia is 3–6 days, with the subsequent formation of an ulcer at the site of infection (i.e., the vector bite).[1,2,4] The patient experiences sudden onset of flu-like symptoms including fever (38–40°C), headache, chills, and generalized aches.[1,2,4] Often swollen lymph nodes develop which resemble the bubo's associated with bubonic plague. Disease without the development of an ulcer is termed glandular tularemia, while disease without either the development of an ulcer or lymphadenopathy is usually referred to as typhoidal or septicaemic tularemia. Oculoglandular, oropharyngeal, gastrointestinal, or pneumonic tularemia are all rare forms of the disease, which occur as a consequence of entry into the host via the relevant mucosal surface.[1]

Septicemic and pneumonic tularemia, when caused by *F. tularensis* subsp. *tularensis,* represent the most severe forms of disease with a typical mortality

rate of 30–60%.[1,2] Both of these forms of disease may develop from ulceroglandular or glandular tularaemia, but primary pneumonic disease is the consequence of the inhalation of bacteria. The clinical features of pneumonic tularemia are quite variable and the disease may present without obvious signs of a pneumonia.[5] Human volunteer studies during the 1950s have provided data on the development of disease in a controlled environment. In these studies the symptoms of pneumonic tularemia developed within 3–5 days of exposure to airborne bacteria and disease was characterized by a fever of up to 40°C. The signs and symptoms of primary pneumonic tularemia include brachycardia, chills, dyspnea, and a nonproductive cough.[5] There may also be headache, sore throat, myalgia, and nausea. There is often hemorrhagic inflammation of the airways that may progress to a bronchopneumonia. Pleuritis is a common feature and enlargement of the hilar lymph nodes is a common radiological feature.[2] Clinical disease may last from a few days to several weeks.[5]

2.2. Animal Models

A number of animal species have been investigated for susceptibility to *F. tularensis* infection. A study published in 1946 reported that *F. tularensis* subsp. *tularensis* (strain SchuS4) was virulent in the mouse, guinea pig, hamster, rabbit, and the cotton rat.[1] The cotton rat was shown to have a high degree of host variation and consequently has received little further attention. A number of subsequent studies have evaluated the infectivity of the four subspecies of *F. tularensis* in mice, guinea pigs, and rabbits. Mice and guinea pigs have been shown to be susceptible to acute disease caused by *F. tularensis* subsp. *tularensis* and subsp. *holarctica*. Rabbits are most susceptible to strains of subspecies *tularensis*.[1] However, the animal species of choice for most studies to date has been inbred mouse strains with BALB/c or C57BL/6 (and genetic mutants of each) mice being most commonly used for pathogenesis and protection studies.

BALB/c and C57BL/6 mice are susceptible to infection with subsp. *tularensis* or subsp. *holarctica*. However, these strains show significant differences in susceptibilities to challenge with the live vaccine strain of *F. tularensis* (LVS).[12] The virulence of *F. tularensis* LVS in mice is also dependent on the route of delivery. This strain is fully virulent when delivered intraperitoneally, but is attenuated when delivered intradermally or subcutaneously.[13] Because *F. tularensis* LVS is virulent in mice when given by some routes, this strain has been used extensively for many studies on the pathogenesis of tularaemia. Conversely, since the intradermal or subcutaneous routes of challenge are analogous to the most frequent route of natural infections, this has led to some debate over the applicability of the model to accurately mimic natural disease caused by fully virulent strains of *F. tularensis*.

The administration of *F. tularensis* LVS into mice by the intradermally or subcutaneous route can result in the induction of protective immunity. However, the nature of this protective response is also dependent on the mouse strain used. Immunized BALB/c mice have been shown to be protected against a subsequent challenge with either *F. tularensis* subsp. *holarctica* or *F. tularensis* subsp. *tularensis* strains, while immunized C57BL/6 mice show little protection against the latter strains.[14,15] The reasons for this have not yet been identified.

2.3. Cellular Pathogenesis

The pathogenesis of tularemia is poorly characterized. However, many insights have come from studies of *F. tularensis* infection of mice, a model generally considered to represent tularemia in humans.[13,16] A major finding was the demonstration that *F. tularensis* is an intracellular pathogen with the ability to replicate within macrophages.[17] In cases of ulceroglandular tularemia it is believed that initial replication of the bacteria occurs locally in the skin within polymorphonuclear leukocytes (neutrophils or PMNs), attracted by chemokines resulting from a pronounced inflammatory response, and resident macrophages. The bacteria are rapidly transported to regional lymph nodes and disseminated by leukocytes[18] by systemic circulation to other organs, especially the spleen, kidney, and liver.[2]

In addition to their ability to parasitize macrophages and neutrophils, it has been demonstrated that *F. tularensis* can also replicate within hepatocytes upon arrival in the liver. The inflammatory response to this foci of infection leads to the recruitment of activated macrophages, NK cells, monocytes, and T cells.[19] These immune cells function to destroy the infected hepatocytes and clear the released bacteria by ingestion of activated macrophages, forming granulomas in the process. The pathogenesis of pneumonic tularaemia is less well understood. However, studies in non-human primates have provided some insight into the likely pathogenesis of the disease. The bacteria are initially confined to the bronchial lymph nodes, and replication appears to occur at this site.[20] Within a few days bacteria are disseminated to the spleen and liver where pyogranulomatous lesions are observed,[21] an outcome also seen following intradermal infection. Similarly, autopsies in fatal cases of tularemia revealed the presence of necrotic granulomas in several tissues including the spleen and lymph nodes.[22] Necrosis of the lung and spleen is also seen in mice following aerosol infection with virulent *F. tularensis,* thus supporting the mouse as a model of tularemia in man.[19]

The mechanism(s) by which *F. tularensis* causes death of some infected individuals is unknown. However, death is often a consequence of organ failure, sepsis, with the subsequent development of systemic inflammatory response syndrome, disseminated intravascular coagulation, and acute and respiratory distress syndrome.[2,5]

2.4. Molecular Pathogenesis

The intracellular niche adopted by *Francisella*, while affording protection from serum immune responses, results in the pathogen's exposure to the potent antimicrobial activity of immune effector cells. However, before entry into phagocytic cells the bacteria must first evade killing by innate serum components such as complement. It has been shown that a capsule deficient mutant of *F. tularensis* LVS is susceptible to the bactericidal effect of nonimmume human sera, although uptake by PMNs of complement opsonized *Francisella* prevents induction of the respiratory burst and killing of the bacteria. In contrast, uptake of *F. tularensis* strain LVS by PMNs leads to killing of greater than 75% of the phagocytosed bacteria.[23] If and when virulent strains of *F. tularensis* express this capsule during infection, and subsequently the mechanism of opsonization and uptake by PMNs and macrophages, remains to be determined.

Bacterial lipopolysaccharide (LPS) is a potent mediator of the proinflammatory response causing infected cells to release cytokines and chemokines such as TNFα, IFNγ, IL-12, CXCL8 and CCL2, and the activation of other innate immune cells. LPS from *F. tularensis* strain LVS is intriguing in its inability to elicit these classic proinflammatory responses. This may be due to an empirical lack of immunostimulatory properties or as a result of binding to a host cell receptor that fails to initiate the production of an inflammatory response. In support of the latter, Telepnev *et al.*[24] have shown that *F. tularensis* LPS does not act as a competitive inhibitor of *E. coli* LPS for the Toll-like receptor (TLR4). Notwithstanding the latter observations, reflection to the gross pathology of *F. tularensis* infection reveals a rapid pronounced inflammatory response, suggesting the existence of an alternative T-cell-independent activation mechanism of macrophages.

The determination of the role of LPS in disease may be further complicated by the observation that some strains of the bacteria can display two types of LPS. The predominant type is a nonstimulatory chemotype (FT LPS) and a second chemotype resembles the LPS produced by *Francisella tularensis* subsp. *novicida* (FN LPS).[25] Analysis of this latter chemotype has revealed that its immunobiological activities are similar to classic immunocompetent bacterial LPS molecules, inducing robust amounts of IL12 and TNFα from mouse macrophages.[26] This raises the possibility that expression of FN LPS during infection may contribute to the pathogenesis of tularemia. However, it has yet to be demonstrated that FN LPS has a similar immunostimulatory effect on human macrophages. Also, concurrent with the requirements to determine the role of capsule in pathogenesis, there is a need to determine when, during infection, the various chemotypes of LPS are expressed.

F. tularensis has been shown to enter macrophages via a cytochalasin B-insensitive pathway with the result that the respiratory burst is not activated.[27] Opsonized bacteria taken up by neutrophils do activate the respiratory burst and while *F. tularensis* LVS succumbs to this bactericidal action, fully virulent

strains of *F. tularensis* subsp. *holarctica* are able to survive. This difference is probably due to the resistance of fully virulent strains to hypochlorous acid, a potent product of the hydrogen peroxide-myeloperoxidase-chloride system.[28] Another antibacterial mechanism of phagocytic cells is the production of nitric oxide (NO). The different chemotypes of LPS can reportedly affect NO production in peritoneal macrophages,[25] suggesting that phase variation of LPS by *Francisella* may modulate this innate immune response. The importance of *Francisella* LPS as a modulator of the immune response and potential virulence factor is supported by studies suggesting that the *Francisella* ABC transporter, *valA,* is important in the assembly of LPS.[29] Furthermore, mutation of this locus renders bacteria susceptible to killing by serum and restricted for growth within macrophages.[30]

Another significant antimicrobial mechanism of phagocytes is the fusion of the lysosome, an organelle containing numerous enzymes capable of degrading a range of macromolecules, with the bacteria-laden phagosome. Other pathogens either prevent phagolysosme fusion, such as *Mycobacteria*, or as in the case of *Listeria monocytogenes*, escape from the phagosome before fusion. *Mycobacteria* prevent fusion of the lysosome by excluding a host vesicular proton ATPase from the phagosome resulting in lack of acidification of the phagosome.[31] In contrast, *Francisella* requires acidification of the phagosome for the sequestration of iron.[32] It has been proposed that this acidification of the phagosome may be a prerequisite for the induction of *Francisella* virulence factors that lead to escape from the phagosome to the cytosol.[33] The mechanism by which Francisella escapes the phagosome is as yet undetermined, but is thought to be distinct from the mechanisms employed by several other intracellular pathogens. *L. monocytogenes* escapes the phagosome by producing a pore-forming listeriolysin; however, no such homologue of this virulence factor has been identified in the genome sequence of *F. tularensis* strain SchuS4 (subspecies *tularensis*).

Analysis of the protein profile of *Francisella* expressed within macrophages identifies very few proteins that are upregulated, suggesting that this pathogen has evolved to be tolerant of the hostile intracellular environment of host phagocytic cells.[34] Of the four proteins that do show upregulation during growth in macrophages, a 23-kDa protein was also shown to be upregulated in response to exposure to oxidative stress, suggesting that this protein's function is related to the adaptation to an intracellular environment. It is interesting to note that there are two copies of the gene encoding this protein, *iglC*, in both *F. tularensis* subspecies *tularensis* and *F. tularensis* LVS, reinforcing the hypothesis that this gene is essential for the intracellular growth of *Francisella*. This hypothesis has been further substantiated by the finding that a derivative of *F. tularensis* LVS, containing mutations in the genes encoding this protein, shows impaired multiplication in a macrophage cell line. This mutant is also attenuated in a mouse model of infection.[35] Another genetic loci that has been implicated as necessary for intracellular growth of *Francisella* is the *mglAB*

locus.[36] It has been proposed that MglAB may be a transcriptional regulator given its high similarity to the *E. coli* regulator SspAB. This idea is corroborated by the observation that a strain of *F. tularensis* subspecies *novicida* harboring a mutation in *mglAB* results in a change in expression of several proteins and precludes intracellular growth.

As with some of the mechanisms of cellular pathogenicity discussed above, *F. tularenis* appears to have a distinct method of ultimately killing its host. It has been shown that the bacteria must multiply intracellularly to induce cytopathogenicity and host cell apoptosis.[37] The mechanism by which apoptosis is effected by *Francisella* is similar to that of the intrinsic apoptotic pathway involving the release of cytochrome C from the mitochondria.[38] However, the upstream mechanisms leading to this programmed cell death remain to be determined.

3. IMMUNITY

3.1. Natural Infection and Immunity

It is generally accepted that recovery from tularemia results in long-lived immunity, with re-infection reported very infrequently.[39] Agglutinating antibodies in serum appear during the 2nd or 3rd week of disease,[5,39] reaching a maximum several weeks later, and remaining detectable in some individuals for at least 10 years after infection.[39] The antibody response that develops after infection is primarily directed toward lipopolysaccharide.[39–41] It is clear that antigens other than LPS are recognized during infection, but some studies have shown that surface proteins may be partially masked by surface polysaccharide.[39] Over the past 15 years a number of protein antigens that are recognized by convalescent sera have been identified,[42,43] work that has been supported recently by the development of proteomic approaches to the identification of immunoreactive antigens (Table II). Antibody to the heat shock protein components Hsp 60 and Hsp10 reportedly predominate,[44] and surprisingly many of the other immunoreactive proteins would also be considered to be cytoplasmically located. The antibody responses that develop to these proteins might be used as the basis of future diagnostic tests for tularemia. However, it is not clear at this stage whether these immunoreactive proteins might be exploited as components of a subunit vaccine.

In parallel, there has been some work to identify antigens able to activate T-cells. At least four outer membrane proteins are able to stimulate proliferation of $\alpha\beta$T-cells taken from individuals who had previously been vaccinated with the live vaccine strain (LVS) of *F. tularensis* or who had previously contracted tularemia.[45,46] These proteins generally appear to stimulate the proliferation of $CD4^+$ T-cells rather than $CD8^+$ T-cells.[47] One of the membrane

TABLE II
Proteins Antigens Reported to be Recognized by Antisera from Individuals Previously Infected with *F. tularensis*

Protein
43 kDa outer membrane protein[42]
Chaperone DnaK[43]
Hsp60[43]
Hsp10[43]
17 kDa lipoprotein (Tul4)[43]
Elongation factor TU[43]
Glycine cleavage system T1 protein[43]
Hypothetical protein[43]
Oxidoreductase[43]
Biotin carrier protein[43]
50S ribosomal protein[43]
Probable bacterioferritin[43]
3-dehydroquinase[43]
Histone-like protein[43]

proteins that is capable of inducing proliferation has been identified as the 17 kDa TUL4 lipoprotein.[48] Hsp10, Hsp60, and DnaK are also capable of causing proliferation of αβT-cells from individuals who have recovered from tularemia.[47] These proteins are associated with the general stress responses of bacteria and are normally considered to be cytoplasmically located. All of these proteins are also recognized by convalescent sera.[43]

There is also sufficient evidence that γδ T-cells are activated in individuals suffering from tularemia.[49] The identity of the antigen(s) which stimulates γδ T-cell activation is not known. In other intracellular pathogens such as *Mycobacterium tuberculosis* nonpeptidic phosphoesters are implicated in this response.[50] Experimental evidence indicates that phosphoantigens also play a role in the activation of γδ T-cells in tularemia patients.[49] Only limited activation of γδ T-cells was seen in individuals who had been immunized with *F. tularensis* LVS, leading to the suggestion that the activation of γδ T-cells may be linked to the virulence of the infecting strain. The significance of the activation of γδ T-cells is not clear. However, the long-lasting recall responses of γδ T-cells appears to be minimal, suggesting that these cells may not contribute to long-term protection against re-infection with *F. tularensis.*[47]

3.2. Live Vaccines

Following Pasteur's demonstration that attenuated viruses could be used as effective vaccines, numerous researchers employed this strategy to the development of vaccines for other pathogens. Perhaps the most notable success

of this approach is the vaccine against tuberculosis, bacille Calmette-Guérin (BCG). Before World War II, similar approaches were undertaken in the former Soviet Union for the development of a vaccine against tularemia. In the 1930s El'bert *et al.* demonstrated protection in a small animal model against a virulent culture of *F. tularensis* following immunisation with an attenuated strain.[51] In 1942, an attenuated strain of *F. tularensis* strain Moscow, was administered to humans with effective protection demonstrated. Development of live vaccines continued in the former Soviet Union, several of which were received by the United States in the 1950s. From one of these strains, a subspecies *holarctica* (Type B), the live vaccine strain (LVS) was developed.[52]

Initial studies with LVS demonstrated that this vaccine was more efficacious in a small animal model when administered as a viable culture,[52] as is the case for BCG. The reason for the increased protection observed with live attenuated intracellular pathogens is not fully understood, although it has been proposed for BCG that active secretion of proteins is required for protection.[53] Although the infectious dose of *F. tularensis* strain SchuS4 is reported to be between 10 and 50 cfu,[8] volunteers immunized with *F. tularensis* LVS were protected against an aerosol challenge with 200 cfu of strain SchuS4.[54] Other studies have shown that *F. tularensis* LVS administered by the respiratory route affords better protection against an aerogenic challenge than intradermal immunisation,[55] a finding also observed with the BCG vaccine.[56] However, *F. tularensis* LVS is usually administered by scarification.

At present LVS, although an effective vaccine against tularemia, is not currently licensed for use. Reasons for this may include mixed colonial morphology and variable immunogenicity, and not least a lack of understanding of the mechanisms of attenuation and protection. However, the finding that an attenuated strain of *F. tularensis* can provide protective immunity suggests that genetically defined and rationally attenuated mutants are a feasible prospect. Such a mutant should be avirulent, be able to replicate *in vivo*, but have a limited ability to survive, ensuring that a protective immune response develops without causing disease. Indeed, for other pathogens, strains containing mutations in genes of essential biosynthetic pathways are already being considered as potential vaccines.[57] The generation of rationally attenuated auxotrophic mutants is favorable, as it has been proposed that their limited replication would allow their administration to immunocompromized hosts without the threat of disease.[58] Many investigators have targeted genes involved in the purine biosynthesis pathways for the construction of rationally attenuated mutants. Analysis of the *F. tularensis* SchuS4 genome sequence indicates that genes encoding all of the enzymes in this pathway are present, but the functionality of this pathway has not been confirmed experimentally. Other genes, which play a role in the growth of *F. tularensis* in macrophages, might also be targeted for the construction of rationally attenuated mutants and a more detailed analysis of the genome sequence may reveal other gene targets for inactivation.

3.3. Subunit Vaccines

At around the same time as the Soviet Union was developing live attenuated vaccines, researchers in the United States suggested that immune serum could be used as a prophylactic treatment of Tularemia in humans. Subsequently, Lee Foshay investigated the possibility of using killed *F. tularensis* cells as a vaccine by virtue of its ability to induce a humoral immune response. Studies in mice, nonhuman primates, and also in humans did demonstrate low level protection against disease,[59] although the reactogenicity of killed whole cell vaccines and the more favorable protection studies with live attenuated strains stemmed further research into the development of killed whole cell vaccines. Nevertheless, the identification of the components of *F. tularensis* responsible for the induction of a protective response, either after immunisation with LVS or natural infection, has been the focus of several studies over the past 50 years.

To date the only protective antigen of *F. tularensis* identified is LPS. Immunization with LPS provides protection against low virulence strains of *F. tularensis*, but is less effective against *F. tularensis* subsp. *tularensis*.[60,61] The lack of protection against high virulence strains following immunization with LPS is thought to result from the requirement of T-cell-mediated immunity for protection.[39] Thus, the development of a subunit vaccine against tularemia can be envisaged as containing LPS coupled with antigen that is capable of eliciting cellular immunity. The number of *Francisella* antigens reported as being able to induce a cellular response is limited. T-cells taken from humans that have been immunized with the LVS vaccine showed proliferation to polypeptides of *Francisella*, having relative molecular weights of 61, 40, 37, 32, 17, and 17.5 kDa.[45] Only the 17-kDa protein and FopA, a 43-kDa protein recognized by convalescent sera, have been evaluated as protective subunits in the murine model of disease. Although both are immunogenic, this response did not provide protection against disease.[62,63]

A method currently employed for the identification of protective subunits uses a novel *in silico* approach for the identification of putative vaccine antigens from genome sequence data.[64] Similar approaches have successfully been used for the identification of potential subunit vaccines for extracellular pathogens. These approaches coupled with the recent completion of the *F. tularensis* strain SchuS4 genome sequence raises the possibility of the identification of new proteins that could be included in a subunit vaccine. In addition, for many pathogens there is a dichotomy that virulence factors are also protective antigens. Identification of virulence determinants of *Francisella* may also add to the arsenal of potential subunit vaccine candidates. How these antigens should be delivered is a major factor contributing to the development of subunit vaccines. Classically, the protein antigen is purified from host bacteria expressing the protein from a plasmid containing its corresponding gene, the purified protein is then administered with a suitable adjuvant. Developments,

to enhance immunogenicity, include administering the gene of the antigen on a plasmid that is recognized by the vaccinee, with subsequent expression of the antigen *in vivo*.[65] Similarly, the gene subunit antigen may be administered in the context of a live attenuated vaccine that invokes an immune response similar to that required for protection against *Francisella*.[66]

3.4. Mechanisms of Protection in Adaptive Immunity

The design of effective new tularemia vaccines requires an understanding of the mechanisms of adaptive immunity that contribute to protection. In humans immunization with LVS leads to protection against virulent tularemia infection and, although the immune responses stimulated have been studied,[67,68] those mechanisms essential for protection are unknown. A murine model of immunization provides a convenient experimental system that can be manipulated to identify these essential protective components in a mammalian system.

The role that antibody plays in protection against disease remains controversial. The adoptive transfer of antibodies has been shown to protect mice against attenuated strains of either *F. tularensis* subsp. *tularensis*[69] or the attenuated *F. tularensis* subsp. *holarctica* strain LVS.[16,70,71] In contrast, no protection has been seen against fully virulent strains of *F. tularensis* subsp. *tularensis*.[39,69] However, in experiments using a low dose challenge of a virulent isolate of *F. tularensis* subsp. *holarctica*, a reduced bacterial burden in the liver and spleens of B-cell-deficient mice following administration of LVS-specific antibody has been demonstrated.[71] Antibodies to LPS have conferred passive protection in mice against challenge with *F. tularensis* LVS, but not against the *F. tularensis* subsp. *tularensis* strain SchuS4.[60] The utility of anti-LPS antibodies was also seen in immunization trials with the O-antigen of *F. tularensis* LVS, which successfully protected against challenge with a fully virulent strain of subsp. *holarctica*, but gave no protection against challenge with a subsp. *tularensis* strain.[61] The role of specific antibody in protection against intracellular pathogens has traditionally been regarded as limited due to the protection from effector mechanisms afforded by the intracellular niche of the pathogen. However, more recent papers review several mechanisms by which antibody may act on intracellular pathogens.[72,73] Various mechanisms by which antibody may protect against tularemia infection have been suggested. LVS and a virulent strain of *F. tularensis* subsp. *holarctica* have both been shown to be susceptible to opsonin-dependent intracellular killing by human polymorphonuclear leukocytes in an *in vitro* assay.[74] In the LVS challenge model, efficacy of passive antibody protection has been shown to be dependent on cellular immunity since no protection was observed in mice deficient in interferon gamma, CD4+ or CD8+ T cells.[75] Thus, the evidence so far suggests that at least in mice antibody is a mechanism of protection against attenuated strains and virulent strains of subspecies *holarctica*, but not against strains of subspecies *tularensis*. The role of

antibody in protection of humans should not be discounted. Nonetheless, as for other intracellular pathogens, T-cell effector functions are likely to be the major component of resistance to infection.

The role of T cells in protection against tularemia is dependent on the animal model used. Several studies have demonstrated that mice immunized with either LPS or *F. tularensis* LVS can survive a subsequent challenge with LVS after depletion of CD4+ and/or CD8+ T cells, although T cells are required for clearance of the challenge.[60,76,77] The role of T cells, including the Thy1.2+CD4-CD8- population, in this model has been reviewed extensively by Elkins *et al.*[78] However, mice immunized with LPS followed by an LVS boost and challenged with the fully virulent *F. tularensis* subsp. *tularensis* strain SchuS4 did not survive when depleted of CD4+ and/or CD8+ T cells.[60] The absolute requirement for T cells in this latter experiment illustrates the difficulty of assessing the importance of a mechanism of protection when using attenuated strains.

CONCLUSIONS

Although *F. tularensis* is one of the most infectious pathogens known, very little is known about the pathogenesis of disease or virulence mechanisms. The origins of this pathogen are not clear~~th~~ere are apparently no close relatives and there may therefore be few parallels with other pathogens which can be drawn on to inform future work. Notwithstanding this, it is likely that significant progress will be made in understanding the biology of this organism in the near future. The determination of the genome sequence of this bacterium, coupled with the development of methods for the construction of defined allelic replacement mutants will support this work. Several important questions need to be addressed. Do similar mechanisms of virulence operate in disease caused by inhalation and vector-borne delivery of the bacteria into the host? What are the mechanisms that allow the bacteria to grow within host cells and to spread from cell to cell, and what is the molecular basis of the clear differences in virulence of the four subspecies of the bacterium? Two approaches to the development of a vaccine seem feasible. Firstly, it may be possible to construct rationally attenuated mutants. The feasibility of this approach is supported by previous clinical experiences with the LVS strain in humans. However, a longer-term goal may be to devise a subunit vaccine.

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