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Francisella tularensis vaccines

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ABSTRACT

Francisella tularensis has attracted attention historically as a biological weapon, due to its high infectivity in aerosols, and the severity of disease in humans. There is no licensed vaccine currently available, although an attenuated live vaccine strain (LVS) was identified in the middle of the last century and has been successfully used to protect humans. Efforts are underway to determine the basis of attenuation of LVS, and to understand the immunity required for protection. Alternative approaches to produce subunit vaccines and defined attenuated strains are also in progress. However, the limitations of animal models may make licensing a candidate vaccine challenging.

Francisella tularensis causes the disease tularemia, a debilitating and potentially fatal disease of man. Although naturally a pathogen of rodents and lagomorphs, humans can be accidentally infected. The presentation of disease and the severity is influenced by the dose an individual is exposed to, route of infection and strain. There are three accepted subspecies of *F. tularensis*: *tularensis*, *holarctica* and *mediasiatica*. There has also been a suggestion to include *Francisella novicida* as an additional subspecies [1], so this nomenclature has also appeared in publications but has yet to be universally accepted. Strains of *F. novicida* are less virulent than *F. tularensis*. Subspecies *holartica* accounts for most natural cases of tularaemia in man, but subspecies *tularensis* is the most virulent for man. *F. tularensis* can be transmitted from animals to man by the bite of an arthropod or insect vector, which gives rise to ulceroglandular tularemia, as can infection through cuts or abrasions while handling infected carcasses: this is particularly relevant for hunters in endemic areas. Ulceroglandular disease is the most common form of natural cases of tularemia. However, there are also ocular, oropharyngeal and inhalational presentations, but these are much rarer than ulceroglandular disease. The inhalation of infectious aerosols gives rise to the most acute form of tularemia, with a case fatality rate of up to 30%, but this can be reduced to around 2% with timely antibiotic therapy [2]. Tularemia responds well to timely antibiotic therapy, but regimens are prolonged, and premature suspension of therapy can result in relapse. Inhalational tularemia is reported in endemic regions most commonly in farmers and landscape gardeners. The World Health Organisation recognised the risk of deliberate release of *F. tularensis*, and undertook modelling to evaluate the impact of the use of this pathogen [3]. It was calculated that 50 kg of bacteria released over an urban area would result in 250,000 cases of disease, 25,000 of whom would need hospitalisation, and 2500 would die despite antibiotic therapy being available. However, even where individuals survive infection, tularemia can be a highly debilitating illness lasting many weeks. Therefore, there is a pressing need for a safe effective vaccine to protect against inhalational tularemia, both for high risk populations and in the event of a deliberate release.

Various approaches have been employed to develop a tularemia vaccine. Historically, crude culture extracts were evaluated, but these were ineffective and reactogenic [4]. The induced immunity was able to reduce the severity of ulceroglandular and typhoidal tularemia in volunteers, but many recipients still developed severe disease [5]. In small animal models these preparations failed to provide any discernible protection, and only provided marginal benefit in primates. The lack of efficacy was probably a result of the complex memory immune response required to protect against *F. tularensis*. Although antibody can protect against low virulence strains, a memory T cell response is essential for protection against high virulence strains [6], and this would be unlikely to be induced by these formulations. Similarly, subunit vaccines have failed to achieve good levels of protection against virulent strains. LPS appears to be a key bacterial component recognised by the human immune system, and can induce a protective immune response against low virulence strains and a delayed time to death in immunised mice challenged with high virulence strains [7–9]. The search for other protective antigens to supplement the protection induced by LPS is ongoing. Hopes of developing a subunit vaccine have received a boost as a result of promising results with new adjuvants, such as immune stimulating complexes (ISCOMS) and CpGs. Killed bacteria adjuvanted in this way were able to protect mice against systemic and aerosol challenge [10]. However, this adjuvant system has yet to be evaluated in humans. Some workers have attempted to improve the immune response to subunits using vaccine delivery systems such as attenuated vector strains

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to achieve the correct immune response. An attenuated strain of another intracellular pathogen, *Listeria monocytogenes*, was used to deliver a range of protein antigens for protection studies in mice [11]. Some protection against challenge with highly virulent *F. tularensis* was observed, but survival of mice in a control group dosed with the *L. monocytogenes* vector and subsequently challenged with *F. tularensis* indicated either a level of cross-protection or non-specific immune responses stimulated by the vector. The latter is a strong possibility as it has been shown that non-specific innate immune stimulation can provide some protection against *Francisella*, particularly lower virulence strains [12–14].

A live attenuated vaccine strain was developed in the middle of the last Century (reviewed by [15]). This strain was derived by repeated passage of a Russian vaccine strain on peptone cysteine agar which gave rise to two phenotypic variants distinguishable by their colony colour, grey or blue, when viewed under oblique light. The blue variant was able to induce a protective immune response in mice, whereas the grey variant was overly attenuated and did not induce immunity [16]. The blue variant underwent further manipulations including lyophilisation and serial passage through mice before being designated as *F. tularensis* live vaccine strain (LVS). However, the blue variant could still give rise to the grey variant during batch production of the vaccine, and the grey variant could represent as much as 20% of some preparations [17]. The LVS strain was tested in human volunteers in "Operation Whitecoat", and subsequently used in laboratory workers in whom there was a resultant reduction in laboratory acquired tularemia infections. Scarification and aerosol immunisation with LVS was evaluated [18–20], and although aerosol vaccination induced more solid immunity in volunteers most humans have received LVS by the dermal route. Following scarification, a small lesion develops at the site of inoculation, suggesting that immunization mimics a mild case of ulceroglandular tularemia. LVS bacteria are not detected in blood following scarification, but bacteria can be detected for up to a week at the site of inoculation, indicative of local bacterial replication [21].

The immune response to LVS has been studied in depth, particularly in mouse models (reviewed by [22]), but there is also data from the human volunteer studies and from natural human infections. One key finding from studying human infections was that there is a significant expansion of the circulating Vγ9/Vδ2 T cell population [23]. However, only primates exhibit this specific response as other animals including mice do not possess a homologous cell receptor. Thus, although the murine response to LVS vaccination is well-understood compared to that of humans and other primates, there are limitations to the model. Despite the differences, there are also commonalities in responses: in both humans and mice it has been shown that robust CD4+ and CD8+ responses, supplemented with antibody responses, are induced in response to vaccination (reviewed by [22,24]). In humans, the memory immune response to *Francisella* can persist for many years [25].

Despite being used successfully for decades to immunize large numbers of people, LVS has as yet failed to achieve licensing by regulatory authorities. The reasons for this are complex, but include a lack of understanding of the basis of attenuation, residual virulence, and the issue of the mixed blue/grey phenotype in vaccine lots. Recently, an improved procedure for the production of the LVS under current good manufacturing practice (cGMP) conditions was developed [26]. This cGMP LVS was shown to be 100% blue phenotype, and safe and immunogenic in rabbits. These studies paved the way for human Phase 1 studies which are currently underway, and which may eventually contribute to LVS receiving full approval for use in humans. However, this would need to be supported by an understanding of the basis of attenuation of LVS. The availability of genome sequence data for a range of *Francisella* strains including LVS has facilitated identification of genes inactivated in LVS, but functional in fully virulent strains [27,28]. Two such genes that have been implicated in contributing to attenuation of LVS are *pilA* [29] and FTT0918 [30]. Mutation of these genes either individually or in combination resulted in attenuation of fully virulent strains. In turn, restoration of the genes with functional copies in LVS was sufficient to restore virulence to the same level as wild type virulent strains [31]. This result could provide the basis of a rationally attenuated strain, or facilitate licensing of LVS for human use.

Due to the problems encountered in licensing LVS, there has been much interest in developing a defined rationally attenuated strain that would be more acceptable. Historically, auxotrophs have successfully been evaluated in the development of vaccines against other bacterial pathogens, often with mutations in pathways involved in purine or aromatic amino acid biosynthesis. These biosynthetic pathways are intact in *F. tularensis* [32,33], although it does exhibit other auxotrophies, most notably a requirement for cysteine. However, the degree of attenuation is dependent on where in a pathway the mutation occurs. For example, a *Francisella purA* mutant was overly attenuated, while a *purF* mutant of the same strain retained higher residual virulence [34]. Inactivation of virulence factors has also been used to attenuate pathogens. For many years, *F. tularensis* was regarded as an enigma in this respect, as no classical virulence factors could be identified. Even with the availability of genome sequence data, it is still not known how uses its 1.8 Mbp genome to produce such a dramatic effect *in vivo*. Some key loci have been identified, such as the intracellular growth locus (*igl*) which is essential for intracellular growth and virulence [35,36], but many aspects of the molecular basis of pathogenesis by this intracellular pathogen remain to be elucidated. Molecular approaches have been applied only relatively recently to *Francisella*, mainly due to difficulties in transformation and a dearth of molecular tools. However, these problems have been resolved, and several methods to transform and mutate *F. tularensis* have been developed. However, strain selection needs to be considered careful, as inactivation of genes in one subspecies does not necessarily have the same effect if mutated in another subspecies. For example, when the *pilC* gene of a subspecies *holarctica* strain was inactivated the mutant retained virulence in a mouse model. However, a *pilC F. novicida* mutant showed an increase in virulence [37]. This surprising effect was subsequently shown to be due to a link with secretion of virulence factors. The more virulent *F. tularensis* secrete very few proteins [38], whereas *F. novicida* secretes at least seven proteins [37]. One of these proteins is PepO, the action of which results in vasoconstriction *in vivo*, and thus reduces dissemination. However, *pepO* is mutated and nonfunctional in subspecies *tularensis* and *holarctica*, which may have been an important step in the evolution of this systemic pathogen. Inactivating *pilC* in *F. novicida* would thus have abrogated PepO secretion, and thus facilitated dissemination [37]. This illustration indicates clearly why interesting attenuation targets should be examined in a range of strains, and also that the molecular interactions cannot always be predictable.

As with many biodefence vaccines, any vaccine for prophylaxis of tularemia would likely be evaluated for licensing under the FDA Animal Rule due to the low numbers of natural human inhalational tularaemia cases worldwide. However, the Animal Rule requires an understanding of the mechanism of pathogenesis which is still lacking. In addition, although most *in vivo* studies have been undertaken in mice, the pattern of susceptibility is not the same for mice as for humans: the mouse is much more acutely sensitive to all subspecies of *F. tularensis* than humans [39], indicating that innate immune responses differ, as exemplified by the γδ T cell response described above. In addition, systemic immunisation of man and primates can protect against highly virulent strains of *Francisella*, but this cannot be reproduced in mice [18,39,40]. Thus, extrapolation from murine models to humans is unlikely to be totally predictive. Similarly rabbits and guinea pigs are more sensitive to