MINIREVIEW

Francisella tularensis vaccines

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Abstract

Francisella tularensis is the causative agent of tularemia, a disease which occurs naturally in some countries in the northern hemisphere. Recently, there has been a high level of interest in devising vaccines against the bacterium because of the potential for it to be used as a bioterrorism agent. Previous human volunteer studies have shown that a strain of F. tularensis [the live vaccine strain (LVS)] that has been attenuated by laboratory passage is effective in humans as a vaccine against airborne disease. However, for a variety of reasons it seems unlikely that the LVS strain will be licensed for use in humans. Against this background there is an effort to devise a licensable vaccine against tularemia. The prospects for a killed whole-cell subunit of live attenuated vaccine are reviewed. A rationally attenuated mutant seems the most likely route to a new tularemia vaccine.

Francisella tularensis and disease

Francisella tularensis was first isolated in 1911 in California and later recognized as the cause of tularemia in many countries in the northern hemisphere (Ellis et al., 2002). Outbreaks of the disease, when they occur, are usually relatively localized and rarely exceed 100 cases. However, in the former Soviet Union outbreaks have occurred that involved tens or even hundreds of thousands of cases. Although outbreaks of tularemia are relatively rare today, F. tularensis remains a significant but overlooked cause of disease. Possibly this reflects the similarity of the symptoms of tularemia with other flu-like illnesses, the relatively low mortality rate and the inability of many laboratories to diagnose the disease.

Francisella tularensis has another more sinister association with disease. The relative ease of culture and very low infectious dose by the airborne route (typically 50 CFU) are both features of pathogens which could be used illegitimately to cause disease. Originally developed as a bioweapon in the 1940s and 1950s by both the USA and the former Soviet Union (Mangold & Goldberg, 1999; Dennis et al., 2001) the bacterium is now considered to be one of the pathogens most likely to be used by terrorist groups to attack civilian populations. It is against this background that there is currently a high level of interest in F. tularensis and specifically in the development of vaccines which can be used to protect susceptible human populations.

Of the four subspecies of F. tularensis (Table 1), subspecies tularensis causes the most severe form of the disease, and is most likely to be used as a bioweapon or bioterrorism agent. Although the disease may occasionally prove to be fatal, tularemia is essentially a severe debilitating disease. The symptoms of respiratory tularemia are quite variable, but the disease is usually characterized by the rapid onset of a high fever of up to 104 °C and may be accompanied by brachycardia, severe chills, dyspnoea, nonproductive cough, pleuritic chest pain and profuse sweating (Gill & Cunha, 1997).

Virulence mechanisms of F. tularensis

Work to understand mechanisms of virulence and the pathogenesis of tularemia currently receives significant funding. In recent years there have been a number of significant findings which add to our understanding of tularemia. Francisella tularensis is generally considered to be an intracellular pathogen and much of the work on virulence mechanisms has been carried out using macrophage cell cultures. However, there is increasing evidence that the bacterium is able to colonize other cell types in vivo, including dendritic cells and neutrophils and non-phagocytic cells such as hepatocytes or endothelial cells.
Table 1. Francisella tularensis subspecies

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Geographical location</th>
<th>LD₅₀ dose in humans</th>
<th>LD₅₀ dose in mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tularensis</td>
<td>North America*</td>
<td>&lt; 10</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>holarctica</td>
<td>Mainly Europe, Siberia, Far East, Japan, Kazakhstan &amp; North America</td>
<td>&lt; 10⁸</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>mediastatica</td>
<td>Mainly central Asia and parts of the former Soviet Union</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>novicida</td>
<td>North America and Australia</td>
<td>&gt; 10⁴</td>
<td>&gt; 10³</td>
</tr>
</tbody>
</table>

*There is one report of the isolation of F. tularensis ssp. tularensis in Europe (Gurycova, 1998).

(Conlan & North, 1992; Forestal et al., 2003; McCaffrey & Allen, 2006; Melillo et al., 2006). The differential roles of these different cell types in infection has not yet been established. Therefore, it is possible that studies with macrophages may not fully reveal the complete nature of the host–pathogen interaction. In addition, although growth intracellularly is essential for pathogenesis it is clear that some genes which do not appear to influence growth in host cells directly still contribute to virulence. For example, in mice, the development of infection from peripheral challenge routes is markedly reduced by the deletion of a putative type IV pilin gene but growth within macrophages is unaffected (Forslund et al., 2006). Similarly, a tolC mutant was attenuated in the murine model of disease but growth in macrophages was not affected (Gil et al., 2006).

Significant progress has been made in characterizing the intracellular lifestyle of F. tularensis. Adherence to macrophages appears to be dependent on complement receptors and on the presence of serum that contains complement factor C3 (Clemens et al., 2005). The surface protein FsAP is required for adherence to human lung cells (Melillo et al., 2006). Bacteria are taken up into macrophages via asymmetric spurious loops, a process which requires bacterial carbohydrates (Clemens et al., 2005). After uptake the bacteria become localized in late phagosomes but acidification of the phagosome appears to be blocked (Golovliov et al., 2003a,b; Clemens et al., 2004). In neutrophils phagocytosed bacteria appear to be able to block the respiratory burst (McCaffrey & Allen, 2006) and the superoxide dismutase SodB appears to play a role in the protection of bacteria from oxidative killing in phagocytes (Bakshi et al., 2006) Later, and by an as yet unknown mechanism, the bacteria escape into the cytoplasm of macrophages, neutrophils and possibly other cell types (Golovliov et al., 2003a,b; Clemens et al., 2004; McCaffrey & Allen, 2006). In macrophages the resultant growth of the bacteria eventually results in apoptosis (Lai et al., 2001; Lai & Sjostedt, 2003), which presumably then allows the bacteria to escape from the cell and subsequently to colonize new host cells. The igl operon within the Francisella pathogenicity island (FPI) (Larsson et al., 2005) is strongly implicated in promoting bacterial growth in macrophages and the subsequent induction of apoptosis (Gray et al., 2002; Lai et al., 2004; Lauriano et al., 2004; Santic et al., 2005). Indeed, studies with different strains of F. tularensis have shown that the IgIC protein can be detected in infected murine tissues or in infected macrophages (Golovliov et al., 1997; Twine et al., 2005). Additionally, a range of stress-response proteins and enzymes which might play a role in resisting macrophage killing mechanisms (such as superoxide dismutase and catalase peroxidase) are expressed under these conditions (Golovliov et al., 1997; Twine et al., 2005).

Iron acquisition systems also appear to play a key role in promoting the growth of F. tularensis in macrophages (Fortier et al., 1995). Initial studies suggested that acidification of phagosomes was required for the acquisition of iron by F. tularensis (Fortier et al., 1995), but a more recent study has shown that the bacterium blocks phagosome acidification (Clemens et al., 2004). The findings outlined above would indicate that the bacterium must also be able to acquire iron after escape into the cytosol. Bacteria which have been grown under iron-limited conditions up-regulate a range of genes including a gene cluster alternatively named fslABCD (Sullivan et al., 2006) or fslABCD (Deng et al., 2006) and which appears to be important for biosynthesis of a siderophore which is closely related to rhizoferrin (Sullivan et al., 2006). Interestingly, iron-limited bacteria also up-regulate the iglC and pdpA genes which are located in the FPI (Deng et al., 2006).

Killed whole-cell vaccines

During the 1920s and 1930s there was a significant effort to develop both killed F. tularensis vaccines for the prevention of disease and antisera against F. tularensis for the treatment of tularaemia. Preliminary studies with heat- or formalin-killed cells showed that vaccines made using these methods were highly reactogenic (Foshay et al., 1942; Foshay, 1950). Using these vaccines the daily dosing with small quantities of antigen over a period of several months was required for the development of measurable agglutinin titres. The killed whole-cell vaccine devised by Foshay, which involved acid extraction followed by phenol preservation, was markedly less reactogenic and several thousand individuals were immunized with this vaccine (Foshay et al., 1942). The efficacy of this killed whole-cell vaccine appears to be
questionable. The Foshay vaccine was able to protect non-human primates against death after challenge with up to 740 CFU of *F. tularensis* strain SCHU S4 (subspecies *tularensis*) (Coriell et al., 1948). However, the immunized animals developed many of the clinical signs of infection after challenge, including local necrotic lesions and regional lymphadenopathy. When tested in mice, the Foshay vaccine afforded a similarly low level of protection against virulent strains. Although these animal studies suggest that killed whole-cell vaccines induced only low levels of protection against disease, studies in humans indicated that immunization with these vaccines reduced the number of infections and considerably modified the course of the disease (Foshay et al., 1942; Kadull et al., 1950).

The lack of protection afforded by heat- or chemical-killed formulations might indicate the inability of these formulations to induce good cell mediated immunity (CMI) responses. Additionally, it may be due to destruction of the antigens caused by the severity of the treatments. Gordon et al. (1964) demonstrated that a vaccine killed using ionizing radiation could induce a low level of protection in mice to challenge with *F. tularensis* strain SCHU S4 (Gordon et al., 1964). Although there may be a case for re-examining the potential for killed whole-cell vaccines, there has been no work reported on this subject in the past decade.

**Subunit vaccines**

The identification of the antigens responsible for induction of a protective response, after immunization with the live vaccine or natural infection, has been a focus of research for 50 years. A number of antigens recognized by either immune sera or T cells have been assessed for protective efficacy in animal models. However, to date the only protective antigen identified is lipopolysaccharide. In humans the predominant antibody response is to lipopolysaccharide and animal studies have shown that immunization with lipopolysaccharide induces protection against *F. tularensis* strains of low virulence (Fulop et al., 1995, 2001). Immunization with lipopolysaccharide is less effective at providing protection against strains of *F. tularensis* ssp. *tularensis* (Fulop et al., 2001; Conlan et al., 2002). This is thought to be due to the requirement for T-cell-mediated immunity for protection (Tarnvik, 1989). One option for a subunit vaccine against tularemia would be to conjugate lipopolysaccharide to an antigen capable of eliciting T-cell immunity. A number of proteins have previously been identified as reactive with human T cells following either immunization with live vaccine strain (LVS) or natural infection (Sandstrom et al., 1987; Surcel et al., 1989; Sjostedt et al., 1990). Of these, only a 17-kDa lipoprotein, a 43-kDa outer membrane protein and heat shock protein 60 have been evaluated for protective efficacy in murine models of disease (Fulop et al., 1995; Golovliov et al., 1995; Hartley et al., 2004). Although immunogenic, the immune response to any of these proteins did not provide protection against experimental tularemia in mice.

The recent completion of the *F. tularensis* strain SCHU S4 genome sequence (Larsson et al., 2005) now provides the opportunity to adopt broader approaches to identify putative vaccine candidates. For example, a group at the Purkyne Military Medical Academy in the Czech Republic have used proteomic methods to identify proteins which react with human convalescent sera or sera taken from LVS-immunized mice (Havlavorav et al., 2005). Whether these proteins are also effective at inducing CD4+ and/or CD8+ T-cell responses awaits investigation.

**Live attenuated vaccines**

Various strains of *F. tularensis* which are attenuated in humans have been considered and in some cases tested as vaccines against tularemia. Although *F. tularensis* ssp. *novicida* is relatively attenuated in humans, it is not suitable as the basis of a vaccine against tularemia because it fails to induce a protective immune response effective against the more virulent subspecies (Shen et al., 2004). This may be either due to lack of expression of protective antigens or due to lack of stimulation of an appropriate immune response. The use of undefined attenuated strains of subspecies *holarctica* as tularemia vaccines has been most widespread in the former Soviet Union, where they have been used to vaccinate large numbers of people. A derivative of one of these strains – the LVS – has also been used extensively in the West. In human volunteer studies the LVS vaccine has been shown to provide good protection against an airborne challenge with 10 infectious doses of a virulent strain of *F. tularensis* ssp. *tularensis* (Fig. 1) (McCrum, 1961), However, only partial protection against 100 infectious doses and poor protection against 1000 infectious doses was reported (McCrum, 1961). Against this background of relatively modest levels of protection, the introduction of the use of the LVS vaccine in laboratory workers at the United States Army Medical Research Institute of Infectious Diseases was accompanied by a marked decrease in the incidence of laboratory-acquired tularemia (Rusnak et al., 2004).

Although live attenuated strains such as the LVS appear to be effective they have been dogged by problems such as reversion to virulence, mixed colony morphology and variable immunogenicity. Some progress has been made in understanding the basis of attenuation of the LVS. A region of genetic difference between the fully virulent SCHU S4 strain (ssp. *tularensis*) and the attenuated LVS (ssp. *holarctica*) has been identified as including genes usually associated with the production of type IV pili. The LVS lacks type IV pili due to a spontaneous deletion mediated by direct
repeats of 120 bp (Forslund et al., 2006), and this deletion may account in part for the attenuation of the vaccine strain (Forslund et al., 2006). Similarly, an isogenic mutant of a subspecies holarctica strain unable to express PilA was also attenuated (Forslund et al., 2006). The mutant was able to interact with macrophages in cell culture, and survived intracellularly, but was unable to disseminate from the initial site of infection. Type IV pili appear to be important not during the intracellular growth of the organism but rather for attachment to cell surfaces during initial stages of infection. In other bacterial species, including other intracellular pathogens such as *Burkholderia pseudomallei* (Essex-Lopresti et al., 2005) and *Legionella pneumophila* (Kwaik, 1998), type IV pili are associated with virulence.

The success of LVS in reducing laboratory-acquired infections (Burke, 1977) indicates that a live attenuated vaccine may offer the best option in the near term for a licensable vaccine against tularaemia. Such a strain should show a limited ability to survive and replicate in vivo, such that a protective immune response develops but without the potential for the bacterium to cause actual disease. However, research into the development of a live *F. tularensis* vaccine strain has been hampered until recently by difficulties in generating such mutants. The organism is difficult to transform and there are few tools available to manipulate the genome. However, a method of conjugation has been developed which can be used to generate allelic replacement mutants (Golovliov et al., 2003a, b), rather than previously available methods such as cryotransformation (Pavlov et al., 1996) or electroporation (Anthony et al., 1991; Baron et al., 1995), which had very low efficiencies. The construction of a shuttle plasmid vector stably maintained in both *Escherichia coli* and *Francisella* (Norqvist et al., 1996) facilitated both complementation studies and the development of reporter plasmids (Kuoppa et al., 2001). Transposons Tn10 and Tn1721 have been shown to be unstable in *Francisella* possibly due to bacterial transposase-complementing activity (Lauriano et al., 2003). However, using a modified Tn5 transposon-transposase (Kawula et al., 2004) or a modified Himar1 mariner (Maier et al., 2006) the problems have been overcome, allowing the identification of attenuated insertion mutants. Many of these mutations have been introduced into *F. tularensis* ssp. novicida, which is virulent in mice, less fastidious and grows relatively rapidly in the laboratory. For these reasons it has been argued that it is a useful model organism for identification of attenuating targets in *Francisella*. Alternatively, mutations have been introduced into *F. tularensis* LVS, which is virulent in mice by some routes of administration. Only very recently has the construction and testing of mutants of high-virulence strains, such as SCHU S4, been reported (Twine et al., 2005).

In other pathogenic bacteria, a range of genes have been targeted to devise rationally attenuated mutants, but in general either biosynthetic pathways or, less frequently, gene regulatory systems have been targeted. Experiences with other pathogens indicate that it is difficult to predict the precise degree of attenuation that will accompany gene inactivation. Over-attenuation will not allow limited
survival and growth of the bacterium in vivo, which is necessary for the induction of a protective immune response, whilst under-attenuation will result in disease.

The identification of suitable genes to target for inactivation has been facilitated by the availability of the F. tularensis ssp. tularensis strain SCHU S4 genome sequence (Larsson et al., 2005). Genes in the purine biosynthesis pathway have been targeted by many investigators for the construction of rationally attenuated mutants of other pathogens. However, the point at which the pathway is interrupted has a profound effect on the degree of attenuation of different pathogens. This also appears to be true for F. tularensis and in addition the ability to induce protective immunity appears to be dependent on the subspecies. For example, mutation of the purMCD locus in F. tularensis ssp. holarctica (Pechous et al., 2006) yielded an attenuated and protective mutant. By contrast, purM or purCD mutants of F. tularensis ssp. novicida are attenuated but do not induce protective immunity (Tempel et al., 2006). Irrespective of the ability to induce protective immunity, all of these mutants showed an inability to grow in macrophages (Tempel et al., 2006).

Other workers have targeted other genes for the construction of attenuated and protective mutants. As an intracellular pathogen, the ability to survive and grow within macrophages is a key virulence trait of F. tularensis. So far one pathogenicity island has been identified in F. tularensis, and this island carries genes essential for intracellular growth. The FPI might be one general target for attenuation. The mutant was able to induce protective immunity (Tempel et al., 2006). Irrespective of the ability to induce protective immunity, all of these mutants showed an inability to grow in macrophages (Tempel et al., 2006).

Mechanisms of protection

The design of an effective tularaemia vaccine requires an understanding of the mechanisms of adaptive immunity that contribute to protection. Although it is established that immunization of humans with LVS will protect against tularaemia, and the immune responses stimulated have been studied, the mechanisms essential for protection are unclear. Antibody responses can be used to monitor whether vaccination is successful, but do not correlate with specific lymphocyte responses in vaccinated humans (Tarnvik & Lofgren, 1975). Immune serum is not protective against highly virulent strains of F. tularensis, but can confer protection against low-virulence strains. Rather, it appears that a cell-mediated immune response is required to protect against high-virulence strains. A study using volunteers in the USA showed that immunization with LVS induced proliferation of CD4+ and CD8+ (but interestingly the TCRγδ+ subset primarily) T-cells, natural killer (NK) cells and monocytes (Fuller et al., 2006). The changes in immune cell populations over 14 days paralleled the pattern of activation of the innate immune system following infection. Cytokine levels in these volunteers were below detectable limits, although increased expression of the interleukin-2 receptor was indicative of a proinflammatory TH1-type response.

The potential for antibody to provide protection against intracellular pathogens has traditionally been regarded as limited because of the protected niche within which the pathogen survives in vivo. However, some reports have
indicated several mechanisms by which antibody may act on intracellular pathogens and therefore contribute to protection (Casadevall, 1998; Casadevall & Pirofski, 2003). The role of antibody in protection against F. tularensis and the precise mechanisms that may be involved are still being debated. In the mouse, adoptive transfer of antibodies has provided protection against attenuated strains of both F. tularensis ssp. tularensis (Allen, 1962) and F. tularensis ssp. holarctica strain LVS (Drabick et al., 1994; Stenmark et al., 2003). The administration of antibody before challenge with virulent F. tularensis ssp. holarctica strains appears to have some protective effect. In a study by Stenmark et al. (2003) LVS-specific antibody reduced the level of bacterial burden in spleens or livers of B-cell-deficient mice given a low-level challenge of a virulent clinical isolate. In our laboratory passive transfer of human anti-LVS IgG has protected 2/5 mice from subcutaneous challenge with F. tularensis ssp. holarctica strain HN63 (Fig. 2; K.F. Griffin et al., unpublished results). However, no protection against challenge with F. tularensis ssp. tularensis has been shown (Allen, 1962; Tarnvik, 1989; K.F. Griffin et al., unpublished results). In mice several studies have demonstrated that protection is associated with antibody to lipopolysaccharide (Fulop et al., 2001; Conlan et al., 2002), but a protective contribution by antibody to protein components is also possible.

Therefore, the evidence in the murine model is that antibody can provide protection against low-virulence strains of F. tularensis and, to some degree, against virulent strains of subspecies holarctica, but not against strains of subspecies tularensis. The role of antibody in protection against human disease should therefore not be discounted. However, as for other intracellular pathogens, T-cell effector functions are likely to be the major component of resistance to infection.

A substantial body of work has concentrated on investigating the role of T cells in protection against challenge with LVS in the murine model (Conlan et al., 1994; Elkins et al., 2003; Yee et al., 1996). Mice can survive the initial challenge with LVS even if they have been depleted of CD4+ or CD8+ T cells. However, T cells are required for subsequent clearance of the infection (Conlan et al., 1994; Yee et al., 1996). However, it has been clear from studies with fully virulent strains that T cells are essential for protection even in the initial stages of disease. For vaccine design these data are highly relevant.

In our laboratory, data suggest that the requirement for CD4+ and CD8+ T cells for protection may differ for infections caused by subspecies holarctica or subspecies tularensis. BALB/c mice immunized with LVS, depleted of CD4+ or CD8+ T cells, all survived subcutaneous challenge with 100 median lethal dose (MLD) of the fully virulent F. tularensis ssp. holarctica strain HN63 (Fig. 3). In both groups, 40% mice culled on day 21 postchallenge had cleared the infection in the spleen. Eighty per cent of mice depleted of both CD4+ and CD8+ T cells also survived for 21 days, although they showed no evidence of clearing the infection (unpublished data). However, protection towards challenge with strains of subspecies tularensis, in the murine model, appears to require both CD4+ and CD8+ T cells. In T-cell depletion studies, following LVS immunization, we have shown that mice depleted of CD8+ T cells alone or CD8+ and CD4+ T cells have died at the same rate as nonimmunized controls. In contrast, 40% of the mice depleted of CD4+ T cells survived, with a high bacterial burden, until culled at 21 days (Fig. 3) (unpublished data). Fulop et al. (2001) also showed that mice immunized with lipopolysaccharide, boosted with LVS and challenged subcutaneously with F. tularensis ssp. tularensis strain SCHU S4, did not survive if depleted of CD4+ and/or CD8+ T cells.

An issue for the development of a vaccine against pneumonic tularemia is whether the mechanisms of protection against this route of exposure are the same as for systemically transmitted disease. Although much is still unknown, Wu et al. (2005) have demonstrated that protection in mice immunized with LVS by the intranasal route, followed by intranasal challenge with F. tularensis ssp. tularensis strain NMFTA1, was dependent on both CD4+ and CD8+ T cells. The group depleted of CD4+ T cells died more quickly than in our study, but it is not clear if this is as a result of challenge strain, number of organisms or route of infection.

In summary, although in the mouse the MLD for fully virulent strains of subspecies holarctica and tularensis are usually similar, the T-cell responses required for protection against strains do differ. One issue for vaccine design is in assessing whether this distinction also exists in human disease caused by the two subspecies and hence whether there is a fundamental requirement for a CD8+ T-cell response in immunized individuals. This requirement does appear to be supported by the findings of Gosselin et al. (2005), who demonstrated that NK and CD8+ T cells dominated the human peripheral blood mononuclear cell
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response to LVS and that the CD8+ T-cell response was minimal when LVS was treated with formaldehyde, a formulation known to be ineffective in protecting mice.

Prospects for improved vaccines

Although the live attenuated tularemia vaccines such as the LVS have been used extensively in humans there are concerns that these existing vaccines may not be licensable. Nevertheless, these vaccines provide important proof of the principle that the induction of protective immunity against tularemia is achievable. An improved vaccine against tularemia would not necessarily provide a greater level of protection than that offered by the LVS – several studies have demonstrated the effectiveness of this vaccine in protection than that offered by the LVS – several studies have failed to identify components of the bacterium that are able to induce protection against challenge with F. tularensis ssp. tularensis. In part, the failure to identify protective subunits might reflect the requirement for CD8+ T cells play a role in protection. Clearly, further work is required before a subunit vaccine becomes a realizable prospect.

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