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Immunoproteomic analysis of the human antibody response to natural tularemia infection with Type A or Type B strains or LVS vaccination

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ABSTRACT

Francisella tularensis is pathogenic for many mammalian species including humans, causing a spectrum of diseases called tularemia. The highly virulent Type A strains have associated mortality rates of up to 60% if inhaled. An attenuated live vaccine strain (LVS) is the only vaccine to show efficacy in humans, but suffers several barriers to licensure, including the absence of a correlate of protection. An immunoproteomics approach was used to survey the repertoire of antibodies in sera from individuals who had contracted tularemia during two outbreaks and individuals from two geographical areas who had been vaccinated with NDBR Lot 11 or Lot 17 LVS. These data showed a large overlap in the antibodies generated in response to tularemia infection or LVS vaccination. A total of seven proteins were observed to be reactive with 60% or more sera from vaccinees and convalescents. A further four proteins were recognised by 30–60% of the sera screened. These proteins have the potential to serve as markers of vaccination or candidates for subunit vaccines.

Introduction

Tularemia is a disease in primates caused by the Gram-negative facultative intracellular bacterium, *Francisella tularensis. F. tularensis* has received increasing attention in the last decade due to its potential for use as a bioweapon (Kortepeter and Parker, 1999; Dennis et al., 2001). Several subspecies exist, with the most clinically relevant subspecies denoted *holarctica* and *tularensis*, commonly known as Type B and A strains, respectively (Sjostedt, 2001). The subspecies *tularensis* (Type A) is endemic only to North America. Mortality rates of up to 60% have been reported for untreated human cases of disseminated infection caused by Type A strains of the pathogen (Dienst, 1963). The subspecies *holarctica* (Type B), endemic to both Europe and North America, is associated with lower mortality rates. Type B strains are responsible for almost all European cases of tularemia (Sjostedt, 2007).

A live vaccine strain (LVS) was derived in the 1950s from a Soviet strain type B, S15, and protects humans to some degree against subsequent exposure to Type A strains of the pathogen (Hornick and Eigelsbach, 1966). Human LVS vaccination studies were conducted under the Operation Whitecoat (OW) program in the 1950s. These data showed that LVS administered by scarification was 25–100% effective against aerosol challenge with SCHU S4 (Hornick and Eigelsbach, 1966). All vaccinees were shown to seroconvert to an undefined set of *Francisella* antigens, but no immunologic correlation was established with the protective status of the host. When LVS replaced killed bacteria as the vaccine at the United States Army Medical Research Institute for Infectious Diseases (USAMRIID), the incidence of respiratory infections among at-risk personnel was significantly reduced (Burke, 1977; Eigelsbach et al., 1967).

Due to renewed concerns regarding the threat of bioterrorism, there has been an increased interest in licensing a tularemia vaccine for general use. However, both the absence of a correlate of protection and the unknown mechanisms of attenuation are significant barriers to LVS licensure. Recent studies using the murine model of tularemia show that adaptive host defense against *F. tularensis* is likely mediated by both cell mediated immunity (CMI) and humoral immunity (Tarnvik, 1989; Elkins et al., 2003; Kirimanjeswara et al., 2008). Although CMI is thought to be the most essential mechanism in host defense against Type A *Francisella*, specific antibody responses are mounted during natural *Francisella* infections or following vaccination (Saslaw and Carhart, 1961; Carlsson et al., 1979; Viljanen et al., 1983; Dennis et al., 2001). The humoral immune response will therefore serve as a facile means of screening sera for markers of successful LVS vaccination.

Ethical considerations prevent a repeat of human LVS vaccine efficacy studies, such as those conducted under OW. Instead, we sought to compare the repertoire of antibodies generated by

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humans in response to natural tularemia infection with Type A or Type B strains and vaccination with NDBR lot 11 LVS or DVC lot 17 LVS (escalating dose study). Patients that recover from types A and B *Francisella* infections are rarely reported to show signs of disease following a second exposure, and therefore could be considered a group that is protected from further challenge. This affords the opportunity to compare the repertoire of antibodies between infected, but presumably protected individuals, and vaccinated volunteers whose protective status is unknown.

Materials and methods

Sera used in this study

Four distinct collections of human sera were used in this study, including sera from two groups of human tularemia patients, LVS vaccinated laboratory personnel and clinical trial subjects immunized with LVS (Table 1). For each study group, informed consent was obtained by the study directors prior to serum collection.

The Type B convalescent sera were from patients diagnosed with tularemia in Sweden, where the disease is considered endemic. In total, sera were available from 12 tularemia patients and 3 healthy individuals with no history of tularemia. The Swedish patients were infected with type B strains and the route of infection for the majority of these patients was intradermal.

The Type A convalescent sera were a subset from a total of 59 subjects with tularemia reported on Martha's Vineyard between 2000 and 2006. Approximately 60% of cases were thought to be due to inhalation of the bacterium (Feldman et al., 2003; Matyas et al., 2007). In this study, sera from the first physician visit were available from 12 confirmed Type A tularemia patients.

Two sets of sera from separate human LVS vaccinations were studied. In the first set, at-risk laboratory workers in Sweden were immunized with LVS NDBR101 Lot 11. The set was comprised of five sets of paired pre- and post-vaccination samples and an additional three post-vaccination serum samples. NDBR lot 11 was prepared as per the vial instructions. Briefly, the vaccine preparation was reconstituted in 2.0 mL of water to give a concentration of 2.5×10^9 CFU/mL. A droplet of approximately 20 μL (containing $\sim 5 \times 10^7$ CFU) was administered by scarification using a bifurcated needle to puncture the skin.

The second human vaccinee serum set was from subjects vaccinated with DVC lot 17 LVS (Pasetti et al., 2008), obtained from a Phase I clinical trial carried out at the Baylor College of Medicine, Houston, TX. The vaccine used was manufactured at Cambrex Bio Science, Baltimore, MD, under contract with DynPort Vaccine Company LLC (DVC). The vaccine was administered as described previously (El Sahly et al., 2009). Briefly, the lyophilized vaccine was reconstituted with 0.25 mL of sterile water for injection yielding a vaccine concentration of 1.6×10^9 CFU/mL. The study design and administration of the vaccine were described in detail previously (El Sahly et al., 2009), with dosages of 10^3, 10^5, 10^7 and 10^9 CFU/mL administered by scarification with a bifurcated needle. Five paired sera (pre- and 42 days post-vaccination) and three unpaired sera (post-vaccination) were provided.

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) Western blotting

The protein antigen used in Western blotting experiments was an LPS deficient mutant, SCHU S4 Δ*wbtI*, to prevent interfering immunoreactivity towards the O-antigen of LPS. Bacterial proteins were extracted as described previously, using a method that solubilizes a broad range of cytoplasmic and membrane associated proteins (Twine et al., 2005). Briefly, the SCHU S4 Δ*wbtI* mutant was grown in modified Cysteine Heart Agar (CHA) for 24–36 h at 37°C within a BioSafety (BS) Level 3 containment facility. Bacteria were harvested from plates and lysed using a solution of 5 M urea, 2 M thiourea, 1% DTT, 4% CHAPS, 0.5% ASB-14, as described in our earlier work (Twine et al., 2005). Proteins were separated using immobilized pH gradient strips (IPG), linear pH 4–7, 17 cm (Bio-Rad, Hercules, CA) or linear pH 6–11 (GE Healthcare, Piscataway, NJ), using 100 μg of protein/gel. Much of our previous work with murine sera showed few if any proteins with a pI <4 or >7 to be reactive with sera from LVS immunized BALB/c or C57/BL6 mice. Given the limited amounts of sera available from clinical samples, therefore, the analyses in this study were initially confined to pH 4–7 (Twine et al., 2010). One serum sample from each study set was also screened against antigen separated in the pH range 6–11. Immunoblotting was carried out according to methods previously published by others (Mansfield, 1995) and described in our own work (Twine et al., 2010).

Identification of immunoreactive proteins

Protein spots corresponding to identified areas of immunoreactivity on Western blots were excised from protein stained 2D-PAGE gels and tryptically digested, as described previously (Twine et al., 2010, 2006). The in-gel digests were analyzed by nano-liquid chromatography–MS/MS (Twine et al., 2010). The peaklist files of MS^2 spectra of the excised protein spots were searched against a database (2008.03.10) with 11947 entries consisting of the NCBI reference genomes for seven strains of *Francisella* (NCBI ids: NC_006570, NC_007880, NC_008245, NC_008369, NC_008601, NC_009257, NC_009749) using MASCOT™ (version 2.2.03, Matrix Science, London, UK) for protein identification, as detailed previously (Twine et al., 2010).

Results

Human serum antibody response is directed towards a relatively small number of proteins

The details of sera used in this study are shown in Table 1. Over 95% of the type B tularemia patients, had the ulceroglandular form of tularemia. Sera from each of twelve type B tularemia patients and from three individuals with no history of tularemia were screened by 2D-Western blotting in the pH range 4–7. Representative Western blots are shown in Fig. 1a and b (the complete series of Western blots are shown in Fig. S1), with a total of 31 identified immunoreactive proteins (Table 2, Fig. 1i). Of the three control sera, drawn from volunteers with no history of tularemia, one serum showed low reactivity with the Chaperonin GroEL. Sera from all 12 tularemia infected individuals showed intense reactivity with the Chaperonin GroEL (FTT_1696). The protein dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (FTT_0077) was immunoreactive with 11 of the 12 patient sera screened but none of the control sera. Due to limited volumes or sera, a single serum sample from patient 1671 was screened against antigen separated in the pH range 6–11 (Fig. 2a). Some background interference was observed in the low MW region of the blot, and a single area of immunoreactivity was observed in the basic high molecular weight region. This protein was not able to be identified and is indicated by arrows in Fig. 2e. Further to this, the proteins 50S ribosomal protein L1/L12 (FTT_0143), hypothetical membrane protein (FTT_1778c), and acetyl CoA carboxylase (FTT_0472) were immunoreactive with 8 or more patient sera screened and with none of the control sera. Of note, the proteins FTT_1778c and FTT_0143 focus to discrete spots on 2D-PAGE within close proximity of one another. In some cases the immunoreactivity