Characterization of lethal inhalational infection with \textit{Francisella tularensis} in the common marmoset (\textit{Callithrix jacchus})

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The intracellular Gram-negative pathogen \textit{Francisella tularensis} is the causative agent of tularemia and is prevalent in many countries in the northern hemisphere. To determine whether the common marmoset (\textit{Callithrix jacchus}) would be a suitable non-human primate model of inhalational tularemia, a pathophysiology study was undertaken. Ten animals were challenged with \(\sim 10^2\) c.f.u. \textit{F. tularensis} strain SCHU S4 (\textit{F. tularensis} subsp. \textit{tularensis}). To look for trends in the infection, pairs of animals were sacrificed at 24 h intervals between 0 and 96 h post-challenge and blood and organs were assessed for bacteriology, pathology and haematological and immunological parameters. The first indication of infection was a raised core temperature at 3 days post-challenge. This coincided with a number of other factors: a rapid increase in the number of bacteria isolated from all organs, more pronounced gross pathology and histopathology, and an increase in the immunological response. As the disease progressed, higher bacterial and cytokine levels were detected. More extensive pathology was observed, with multifocal lesions seen in the lungs, liver and spleen. Disease progression in the common marmoset appears to be consistent with human clinical and pathological features of tularemia, indicating that this may be a suitable animal model for the investigation of novel medical interventions such as vaccines or therapeutics.

INTRODUCTION

\textit{Francisella tularensis} is a Gram-negative intracellular pathogen and the causative agent of tularemia. The disease is prevalent in many countries in the northern hemisphere and is infectious by a number of routes (Ellis \textit{et al.}, 2002). Due to its high infectivity and virulence, \textit{F. tularensis} has been developed previously for use as a bioweapon (Dennis \textit{et al.}, 2001) and is considered a Category A agent on the Centers for Disease Control and Prevention threat lists (CDC Emergency Preparedness and Response website; http://emergency.cdc.gov/agent/agentlist-category.asp).

A number of animal models, mainly rodents, have been used to study disease pathogenesis, to understand the host immunology and to assess the efficacy of various therapeutic agents and vaccines. The majority of work to study the pathogenesis of tularemia has used murine models of infection (Conlan \textit{et al.}, 2003; Green \textit{et al.}, 2005). BALB/c mice and C57BL/6 mice are susceptible to low doses (between 10 and 20 c.f.u.) of both \textit{F. tularensis} subsp. \textit{tularensis} and \textit{F. tularensis} subsp. \textit{holarctica} (formally known as type A and type B strains, respectively; Green \textit{et al.}, 2005).

Despite the valuable information obtained from rodent models, there are limitations. Generally, one main limitation of animal models of infectious diseases is the inability to predict human responses to pathogens or therapeutic compounds accurately (Herodin \textit{et al.}, 2005). There are advantages to using non-human primates to model infectious diseases; for tularemia, primates are the only experimental animals that develop the characteristic skin ulcers and lymphadenitis of tularemia, whilst other clinical features reflect human disease more readily than rodent models. There is a need to develop robust non-human primate models of infection to assess potential vaccines and therapeutics.

Marmosets have been demonstrated to be susceptible to tularemia in two naturally occurring outbreaks in captive animals (Posthaus \textit{et al.}, 1998; Splettstoesser \textit{et al.}, 2007).
The reported disease pathology corresponded to experimental infection in other non-human primates. We have demonstrated previously that marmosets are susceptible to experimental inhalational tularemia and develop a lethal infection (Nelson et al., 2009). The aim of the current study was to characterize the progression of the disease in the marmoset to determine the suitability of the species as a model of human infection.

METHODS

Animals. Healthy, sexually mature common marmosets (*Callithrix jaccus*) were obtained from the Dstl Porton Down breeding colony and housed in female and vasectomized male pairs. Adult animals were aged between 19 and 84 months old and weighed between 354 and 506 g at the time of challenge. The animals were allowed free access to food and water, as well as environmental enrichment. Prior to use in challenge studies, animals were surgically implanted intraperitoneally with a Remo 200 device to record core body temperature (Tc), under general anaesthesia (ketamine/medetomidine/isoflurane). Data were transmitted from the devices at 10 s intervals to locally placed antennas and relayed to receivers. Data were analysed using eDacq software to provide a real-time and recordable Tc.

All animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 and the Codes of Practice for the Housing and Care of Animals used in Scientific Procedures 1989. Following challenge with *F. tularensis*, all animals were handled under UK Advisory Committee on Dangerous Pathogens animal containment level 3 conditions within a half-suit isolator compliant with British Standard BS5726.

Bacterial strain and culture. Glycerol stocks of *F. tularensis* strain SCHU S4 were obtained from DynPort Vaccine Company LLC. Bacteria were recovered from the vial onto blood cysteine glucose agar (BCGA) plates and incubated at 37°C for 24 h, prior to recovery into PBS (pH 7.3). The OD590 of the suspension was adjusted to 0.1, equivalent to approximately 1 x 10^8 c.f.u. ml^-1, 1 ml of which was used to inoculate 100 ml modified cysteine partial hydrolysate broth. The broth was shaken at 180 r.p.m. for 48 h at 37°C. Prior to challenge, the OD590 of the culture was adjusted to 0.1 and serially diluted in PBS to the appropriate concentration for challenge. Viable counts were performed by subculture onto BCGA retrospectively. Plates were incubated at 37°C for 72 h prior to enumeration. All bacteriological procedures were carried out under UK Advisory Committee on Dangerous Pathogens animal containment level 3 conditions in Class 3 microbiological safety cabinets compliant with British Standard BS5726.

Challenge. The marmosets were anaesthetized with 25 mg ketamine kg^-1 administered intramuscularly prior to exposure and were challenged in pairs by the airborne route (Lever et al., 2008; Nelson et al., 2009). Briefly, a Collison nebulizer containing 20 ml *F. tularensis* and three drops of Antifoam 289 (Sigma) was used to generate aerosol particles of approximately 1–3 μm. The aerosol was conditioned in a modified Henderson apparatus (Druett, 1969). Marmosets were placed in a head-only exposure chamber (plethysmograph tube) and exposed for 10 min to a dynamic aerosol maintained at 50–55 % relative humidity and 18–20 °C. The total accumulated tidal volume for each animal during challenge was determined by whole-body real-time plethysmography with a Fleisch pneumotachograph (EMMS). The concentration of the aerosol cloud was quantified after sampling from a sample port into an all-glass impinger (AGI-30; SKU) by serial dilution and plating onto BCGA.

Characterization of the disease. Ten marmosets were challenged in pairs with *F. tularensis* strain SCHU S4 by the airborne route (Table 1). Pairs of animals were sacrificed at 0, 24, 48, 72 and 96 h post-challenge to investigate disease progression trends. Blood was removed from marmosets immediately after culling by cardiac puncture for assessment of bacteremia, clinical chemistry, haematology and immunology. Post-mortem examinations were performed on all animals upon culling; organs were removed and assessed for bacteriology, immunology, gross pathology and histopathology.

Bacteriology. Bacterial loads in the blood, liver, spleen, kidneys and lungs were assessed for all animals. Sacrificed animals were dissected, the organs were removed aseptically and the organ weight was determined. Organs were homogenized in 5 ml PBS using cell sieves and a plunger. Organ homogenates and blood were serially diluted in PBS and the appropriate dilutions were subcultured onto BCGA plates, which were incubated at 37 °C for 72 h prior to enumeration. Counts were expressed as c.f.u. (g tissue)^-1 or c.f.u. (ml blood)^-1.

Cytokine and chemokine determination. Blood and organ homogenates were also processed for immunological analysis. For cytokine and chemokine analysis, the cell suspension supernatants were collected and stored at -80 °C until assayed. A range of cytokines appropriate for qualification of an innate response was measured. The cytokines were assayed using a Cytometric Bead Array Flex Set (BD Biosciences) according to the manufacturer’s instructions. Briefly, cytokine standards were reconstituted in assay buffer diluent (buffered protein solution) and serially diluted. Ten

<table>
<thead>
<tr>
<th>Time of culling (h)</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Body weight (g)</th>
<th>Inhaled accumulative volume (l)</th>
<th>Aerosol concn (c.f.u. 1^-1)</th>
<th>Inhaled dose (c.f.u.)</th>
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Table 1. Animal and exposure dose data
microlitres of each cytokine capture bead suspension per test was mixed and transferred to the assay tubes. The test sera or standard dilutions and phycoerythrin detection reagent (50 μl) were added to sample tubes and incubated for 2 h in the dark. The samples were then washed with 1 ml wash buffer (PBS, protein and detergent) and centrifuged. One millilitre of 4 % paraformaldehyde was added to each tube for fixation at 4 °C for 24 h prior to analysis. Cytokine detection was performed by flow cytometry on a BD FACS Canto using BD FACS DIVA and FACS Canto software. Cytokine and chemokine levels were expressed as pg ml⁻¹, except for monocyte chemotactic protein-1 (MCP-1), which was expressed as fluorescence intensities, as, due to the failure of the standard antibody, a calibration curve was not determined.

**Cell type determination.** Cell type was assessed from the fresh cell suspension. A range of anti-human monoclonal antibodies was used for lymphocyte staining according to the manufacturer’s concentrations: anti-CD3 (clone sp34; BD Biosciences), anti-CD4 (clone MT310; Dako), anti-CD8 (clone i8; AbD Serotec), anti-CD11c (clone s-hcl-3; BD Biosciences), anti-CD14 (clone m5e2; BD Biosciences), anti-CD20 (clone B-Ly1; Dako), anti-CD27 (clone m-t271; BD Biosciences), anti-CD56 (clone NCAM16.2; BD Biosciences) and anti-V-2 (15D; Endogen) stain. Single-cell homogenate or whole blood (100 μl) was stained using these fluorescently labelled antibodies, which have previously been reported as cross-reacting with marmoset leukocytes (Brok et al., 2001). After staining for 40 min at room temperature in the dark, red blood cells were lysed using OptiLyse C (Beckman Coulter), washed and resuspended in 4 % paraformaldehyde and kept at 4 °C for 24 h before being analysed on a six-colour BD FACS Canto II using FACS DIVA software. Data for 10000 events of appropriate proportions for live leukocytes were collected. Briefly, leukocytes were defined by appropriate location and the following staining: CD3⁺ as T cells, CD3⁺ CD8⁺ CD56⁻ as activated cytotoxic T cells, CD3⁺ CD68⁻ as CD4 T cells, CD3⁺ V-2⁺ CD56⁺ as activated γδ T cells, CD3⁺ CD56⁺ CD8⁻ as natural killer (NK) T cells, CD3⁻ CD56⁺ as NK cells, CD14⁺ CD11c⁻ as neutrophils, CD14⁺ CD11c⁺ as macrophages and CD20⁺ as B cells. Cell type levels were expressed as a percentage of the viable population.

Due to the small group sizes, statistical analysis was not possible. Only increases in percentages of cells to twice that of the normal mean (obtained from three uninfected animals) and outside the ranges are reported.

**Histopathology.** Tissues were fixed in 10 % neutral buffered formalin and processed for paraffin wax embedding using standard techniques. Thin sections (5 μm) were cut and stained with haematoxylin and eosin for histopathological analysis.

**Haematology, clinical chemistry and electrolytes.** Blood was collected at defined time points into tubes containing EDTA and key parameters were measured by use of a laser flow cytometry-based haematological analyser (LaserCyte; IDEXX Laboratories): total white blood cell count, platelet count, packed cell volume (haematocrit) and haemoglobin. Plasma (lithium heparin) concentrations of albumin, alkaline phosphatase, total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase, glucose and creatinine were determined using a ‘dry-slide’ technology biochemistry analyser ( VetTest; IDEXX Laboratories). An ion-selective electrode-based electrolyte analyser (VetLyte; IDEXX Laboratories) was used to measure K⁺ and Na⁺ concentrations. Post-challenge data were compared with the mean data from the same group of animals analysed pre-challenge.

**Statistics.** Pearson’s correlation analysis was used to determine the relationship of gender, body weight, time to death and inhaled dose.

**RESULTS AND DISCUSSION**

The aim of this study was to examine disease progression trends in marmosets following an inhalational challenge with *F. tularensis*. Ethical considerations necessarily limited group sizes, but the study design none the less provided useful data. Animals were challenged with ~10² c.f.u. *F. tularensis* and culled at specific time points post-challenge (Table 1). Previous data have shown that marmosets challenged in this dose range die between 4.5 and 7 days post-challenge and that there is no statistical correlation between the age, weight or gender of marmosets and susceptibility to inhalational tularemia (Nelson et al., 2009). The course of infection was described in terms of temperature profile, clinical signs of infection, cytokine profile (data not shown), bacteriology (Fig. 1), immune cell populations (Fig. 2), histopathology (Fig. 3), and haematology and clinical parameters.

**Early disease (0–48 h)**

Mild pathological features, including mild enlargement of the inter-alveolar septa, were observed in the lungs of one animal culled immediately after challenge due to the aerosol exposure process. Other minimal foci of inflammatory mononuclear cell infiltrations in the kidney and liver were also considered to be background pathology.

During the early disease, animals appeared normal with no apparent clinical signs or changes in behaviour and had a normal Tc. On post-mortem, some slight haemorrhagic lesions were apparent in the lungs of both animals at 24 h post-challenge, which may have been as a result of the blood withdrawal via cardiac puncture. There was no evidence of gross pathology in any other organs. At 48 h post-challenge, both animals exhibited splenomegaly, with slight hepatomegaly in the male and enlarged lungs in the female. Minor histopathology was observed in all these animal tissues, such as small areas of emphysema in the lungs, as well as other signs considered to be background histopathology.

At 24 h post-challenge, bacteria were recovered only from the lungs of animals, with increasing numbers by 48 h post-challenge, when there were also low levels of bacteria recovered in the spleen (Fig. 1).

Baseline haematological and biochemical parameters for the marmosets used in this study were comparable to previously reported data (Yarbrough et al., 1984). Notable blood parameter changes at 24 h post-challenge included: slightly raised neutrophil percentage counts to 57.5 % (but not total white blood cell counts) compared with pre-challenge baselines of 42 %, a lowered platelet count to 434 × 10³ (baseline value of 499 × 10³) and significantly elevated glucose concentrations to 340 mg dl⁻¹ (baseline value of 197 mg dl⁻¹), plus altered liver enzyme results (elevated ALT to 89 U l⁻¹ and elevated AST to 586 U l⁻¹ compared with baseline values of 11 and 134 U l⁻¹, respectively). Changes in blood parameters at 48 h post-challenge included elevated bilirubin to 0.8 mg dl⁻¹ (baseline value of 0.4).
In order to assess the immune response in marmosets, anti-human antibodies were successfully used to detect and identify marmoset cell types and cytokines as described previously (Brok et al., 2001). Flow cytometry analysis showed an increase in MCP-1 levels (not shown) and the proportion of neutrophils in the lung at 24 h post-

![Graph showing bacterial load isolated from selected marmoset organs at different times post-challenge with ~10^5 c.f.u. inhalational F. tularensis.](image1)

**Fig. 1.** Bacterial load isolated from selected marmoset organs at different times post-challenge with ~10^5 c.f.u. inhalational F. tularensis \[n=2 \text{ for all time points, except terminal animals where } n=6 \text{ (samples obtained from a previous study; Nelson et al., 2009).}\]

![Graph showing proportion of live cells at different times post-challenge.](image2)

**Fig. 2.** Cellular populations isolated from selected marmoset organs at different times after challenge with ~10^5 c.f.u. inhalational F. tularensis \[n=2 \text{ for all time points, except naïve control animals (baseline values) where } n=3 \]. (a) Lymphocyte populations in the lung. (b) T-cell summary.
challenge (Fig. 2a). By 48 h post-challenge, an increase in the number of neutrophils in the lungs (Fig. 2a) and blood (not shown) was observed to approximately 30%. Increases in the percentages of NK cells and macrophages (57 and 13%) were also observed in the lungs (and an increase in T cells to 11%) (Fig. 2a), with an increase in T and B cells in the spleen to 23 and 19%, respectively (not shown). There was also an increase in the percentage of CD8^+ T cells in the spleen and lungs to 13 and 11%, respectively, with a slight increase in the proportion of the γδ T-cell population in the lung to approximately 3% (Fig. 2b).

**Overt disease (72 h onwards)**

No overt clinical signs were apparent in any of the animals until 96 h post-challenge, when they were subdued in nature, with some slight piloerection. Animals that died from infection or were culled due to severe infection (between 4.5 and 7 days post-challenge) generally presented with a progression of overt clinical signs of infection including lethargy, piloerection, and unsteadiness or slowness of movement (Nelson et al., 2009). The first indication of infection in the marmoset is a rapid increase in body temperature, which occurred in this study at 72 h post-challenge. At this time, the mean Tc increased from 38.8 to 39.6 °C for the male and from 38.5 to 40.4 °C for the female. The temperatures continued to increase, and by 96 h post-challenge the Tc of the animals remained consistently high (greater than 40 °C) for at least 2 days. The time to onset of fever in humans is similar and has been shown to occur at 3–6 days post-exposure (Evans et al., 1985; Saslaw et al., 1961). The increase in temperature at this time in the marmoset infection coincided with a number of other factors. There was a rapid increase in the number of bacteria isolated from all organs tested (Fig. 1), a more pronounced gross pathology and histopathology, and an increase in the immunological responses. The bacteria spread from the lungs to the reticuloendothelial system (initially the spleen, followed by the liver), with significant numbers in all organs assessed at 96 h observed in the marmoset, as has also been observed in murine and human infections (Conlan et al., 2003; Gill & Cunha, 1997).

**Histology.** The small areas of lesions observed at earlier times in the lung, liver, spleen and lymph tissue developed...
into focal areas of pyogenic lesions (particularly pyogranulomatous pneumonia) by 72 h post-challenge (Fig. 3a). This developed to pyogranulomatous pneumonia in the lungs by 96 h, with multifocal to coalescent inflammation apparent in one animal (Fig. 3b). Animals also exhibited necrosis in the draining lymph nodes (lymphadenitis), hepatitis, splenitis and interstitial pneumonia by 96 h post-challenge (Fig. 3c, d). The primary infiltrating cell type was neutrophils, but lymphocytes, macrophages and plasma cells were also identified. This pathology, including pyogranulomatous pneumonia, interstitial pneumonia, organ necrosis and the influx of neutrophils and macrophages into tissue, is also seen in human infection (Avery & Barnett, 1967; Martin & Marty, 2001; Syrjala et al., 1986). Other pathological features reported in human infections were also observed in the marmosets, including thrombosis, giant cells and lung oedema (Martin & Marty, 2001; Syrjala et al., 1986).

Immunology. The type of immune response elicited by the marmosets in response to F. tularensis infection was indicated by the cytokine levels detected. Generally, cytokine levels increased with disease progression, with the highest levels in animals that were culled as a result of severe infection (terminal animals). Levels of MCP-1 were detected for the first time in the spleen, blood and lungs at 72 h post-challenge (1.1 x 10^3, 1.15 x 10^3 and 1 x 10^3 pg ml^-1, respectively). The levels further increased in all organs until just prior to death, reaching more than 5000 pg ml^-1. By 96 h post-challenge, there was an increase in the concentration of macrophage inflammatory protein 1x (MIP-1x), MIP-1β, MCP-1, interleukin-6 (IL-6), RANTES and IL-1β present in all organs. The presence of MIP-1x and IL-6 was first observed in animals prior to death. The detection of IL-6 in animals shortly before death is also observed in mice following inhalational challenge (Conlan et al., 2008). The cytokine response observed was consistent with the attraction of macrophages, immature dendritic cells, basophils and lymphocytes to sites of infection and the production of fever, sepsis and tissue necrosis.

The first cell types to reach the sites of infection were the neutrophils and NK cells at 24 h post-challenge, followed by macrophages, NK cells and T cells at 48 h post-challenge. By 72 h post-challenge, there was a decrease in the percentage of neutrophils in the blood and lungs to 23 and 3 %, respectively (Fig. 2a and data not shown), which returned to within normal parameters by 96 h post-challenge. The role of neutrophils in F. tularensis infection is unclear; it has been shown that they are essential in murine survival by reducing the bacterial burden in organs (Sjöstedt et al., 1994). This is despite evidence to suggest that, although F. tularensis is readily taken up by neutrophils, they are unable to kill the pathogen. This study indicated that, despite a large increase in the neutrophil population at 48 h post-challenge, there was no apparent control of bacterial numbers; indeed, the bacterial load in each organ tested rapidly increased after this time. The rapid increase in the neutrophil population reported here in the early response to tularaemia infection has been shown in BALB/c mice in response to type B but not type A strains (Hall et al., 2008). Failure of the F. tularensis strain SCHU S4 to repress neutrophil recruitment may reflect a basic difference between the murine and a more resistant animal model, indicating that the marmoset may provide a more representative model for the human disease. The reduction in neutrophil numbers at 72 h post-challenge may be due to the destruction of these cells, particularly in the lungs.

At 72 h post-challenge, there was also a decrease in the percentage of all cell types in the lungs including a decrease in NK cells to 2 %, and a decrease in T and B cells and macrophage populations to less than 1 % each (Fig. 2a). There was an increase in the T and B cell populations in the spleen to 27 and 29 %, respectively, and also in the blood to 23 and 12 % (data not shown). By 96 h post-challenge, the percentage of most cell types had returned within normal parameters in all organs (Fig. 2a and data not shown). However, there was a further increase in the number of CD8^+ T cells in the spleen and lung, and, most interestingly, there was a large increase in the γδ T-cell population in these organs (Fig. 2b). γδ T cells are known to accumulate at sites of infection and are thought to act as a first line of defence against certain intracellular pathogens. They have been linked repeatedly with Francisella infections in humans (Kroca et al., 2000; Poquet et al., 1998; Sumida et al., 1992). However, it is not clear whether this increase is linked to survival or infection. In the current study, there was no observable increase in the γδ T-cell population in the blood, although marked increases in the spleen and lungs were observed. It is not surprising that the cell numbers initially increased at the site of infection, prior to circulation. Indeed, increases in the γδ T-cell population in the blood of patients suffering from ulceroglandular tularaemia were not detected until 1 week after infection (Kroca et al., 2000).

Blood parameters. Notable changes in the physiological state of the animal included an increase in creatine kinase levels at 72 h post-challenge, plus further evidence of altered liver function (elevated AST in both animals, and raised ALT in the female). By 96 h post-challenge, further changes included elevated white blood cell counts to 14.3 x 10^3 μl^-1 from baseline values of 9.3 x 10^3 μl^-1, a lowered platelet count in the male to 276 x 10^3 μl^-1 from baseline values of 499 x 10^3 μl^-1 and significantly raised plasma glucose and potassium to 685 mg dl^-1 and 11.4 mmol l^-1 from baseline levels of 197 mg dl^-1 and 3.9 mmol l^-1, respectively. In animals that died or were culled due to severe infection, significantly reduced platelet counts and plasma glucose concentrations were noted from 499 x 10^3 μl^-1 and 197 mg dl^-1 to 49 x 10^3 μl^-1 and 29 mg dl^-1, respectively. There were also significantly elevated creatine kinase levels and raised levels of liver
function enzymes (including bilirubin), plus a raised plasma creatinine level. These changes in blood parameters, although limited by the small number of animals studied at each time point, were consistent with the onset of systemic infection. Changes in plasma glucose, creatine kinase, liver function enzymes and platelets, as well as no change to haemoglobin levels, have been observed previously in tularaemia patients (Evans et al., 1985; Kaiser et al., 1985).

In conclusion, experimental acute inhalational tularaemia in the common marmoset appears to be representative of the infection seen in humans. Clinical presentation, bacterial spread, pathology and haematological, biochemical and immunological changes (including increased γδ T cells) are similar between both species. These findings suggest that the common marmoset may be a suitable animal model of inhalational tularaemia, which may be useful for the investigation of novel medical interventions such as vaccines and therapeutics.

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