ORIGINAL ARTICLE

Assessment of low-molecular-weight antioxidants in *Francisella tularensis* infected hosts: comparison of two rodents with different susceptibility to tularemia

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Abstract

OBJECTIVES: Bacterium *Francisella tularensis* is the causative agent of tularemia disease. It is a zoonosis accompanied with high mortality when untreated. Small rodents and hares, in particular, are natural reservoirs of tularemia. Despite physiological similarity of common hosts, tularemia exerts different mortality rates. The pathogenesis of tularemia is still not fully understood. The main pathway is associated with proliferation in macrophages after activation by reactive oxygen species in phagosomes.
DESIGN: A fully virulent strain of *F. tularensis* subsb. *holarctica* was used for infection of laboratory BALB/c mice (*Mus musculus*) and common voles (*Microtus arvalis*) representing murine and microtine species. The total level of low-molecular- weight antioxidants (LMWA) in plasma was assayed by cyclic voltammetry.
RESULTS: It was found that common voles are more resistant to tularemia progression when compared to mice. When LMWA assayed, surprising changes in LMWA levels were found. Both mice and common voles were infected with high dose resulting in overall mortality. While there was a quick depletion of LMWA in plasma in mice, common voles were even able to increase LMWA.
CONCLUSION: It seems that LMWA play an important role in the organism´s protection during tularemia. The ability to compensate the LMWA losses and increase levels of antioxidants in common voles is probably responsible for its lower susceptibility to tularemia.

INTRODUCTION

Francisella tularensis, with four recognised subspecies, is a gram-negative bacterium eliciting the zoonotic disease called tularemia. *F. tularensis* subsp. *tularensis* and *holarctica* are the most virulent subspecies when compared with the nearly avirulent *mediasiatica* and *novicida* (Pohanka *et al.* 2007a). The subspecies *tularensis* is more virulent than *holarctica*. Its geographic distribution however, is restricted to North America except for one isolated instance reported from Central Europe

(Gurycova, 1998). In contrast, the subspecies *holarctica* is widespread over the Northern Hemisphere occurring often in wild animals (Zhang *et al.* 2006). The subspecies *tularensis* can be distinguished from *holarctica* by the fermentation of glycerol and L-citrulline (Olsufjev *et al.* 1959) or by analysing the 16S rRNA structure employing elaborate typing techniques (Forsman *et al.* 1990; Sandstrom *et al.* 1992).

Populations of hares (Pikula *et al.* 2004; Treml *et al.* 2007) and small rodents (Wobeser *et al.* 2007) are considered natural reservoirs of tularemia. Instances of human disease are associated with the activation of endemic foci of tularemia, outbreaks in voles and hares and also with the higher prevalence of F. tularensis in vectors such as ticks (Eisen 2007; Hubalek *et al.* 1996) or mosquitoes (Eliasson *et al.* 2002) and are frequently the result of handling tularemic hares or exposure from farm work (Jenzora *et al.* 2008). Even past historical plagues may have been caused by tularemia spreading from mice (Trevisanato, 2007). The manifestation of tularemia may include multiple forms depending on the route of exposure. Ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal and septic forms are the most frequent (Pullen & Stuart, 1945). The disease is quite progressive and is accompanied by a fever, cough, body aches and weakness, i.e. flu-like symptoms, and lymph node enlargement (Plourde *et al.* 1992; Shapiro & Schwartz, 2002).

Most experimental studies during the last several decades were using the live vaccine strain (LVS) of *F. tularensis* attenuated for humans, thus enabling laboratory work under containment level 3 conditions. Rapid propagation of *F. tularensis* LVS cells in spleen, lung and liver tissue was observed in the BALB/c mouse specimen. *F. tularensis* is a facultative intracellular bacterium, and host cells are invaded within a few hours following the intraperitoneal and intravenous administration of LVS (Fortier *et al.* 1991). *F. tularensis* settles in a phagosome within 30 minutes of infection. This is followed by its escape into the cytoplasm and subsequent propagation (Craven *et al.* 2008). Cytokines such as gamma interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α) collaborate to facilitate the intracellular destruction of *F. tularensis*. Later on, CD4(+) and CD8(+) cells participate in the anti-tularemia adaptive immunity process (Elkins *et al.* 2007) and the production of immunoglobulins M and G (Pohanka, 2007).

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are intermediates involved in the host's defence against various intracellular pathogens including *F. tularensis*. The production of reactive molecular species is induced in macrophages when they are exposed to pro-inflammatory cytokines, such as IFN-γ and TNF-α. After activation, macrophages are capable of arresting bacterial replication (Lindgren *et al.* 2004). *F. tularensis* is exposed to ROS and RNS not only in macrophages but also in other cell types or extra-cellularly in vivo and both *F. tularensis* and *holarctica* subspecies are assumed to be virulent because they are armed with a variety of enzymes that can combat host ROS and RNS-mediated killing mechanisms (Lindgren *et al.* 2007). Hydrogen peroxide and superoxide are one of the most important oxidants found in body (Horvathova *et al.* 2008; Macickova *et al.* 2008). It has been demonstrated that the *F. tularensis* stress response required for its survival in diverse hostile environments is dependent upon the MglA transcriptional regulator of genes which contributes to its virulence by the encoding the *Francisella* pathogenicity island (Guina *et al.* 2007).

The results of some studies suggest that the *F. tularensis* infection confers an oxidative stress upon the target cells, and that many of the host-defence mechanisms appear to counteract this stress (Andersson *et al.* 2006). Therefore, a shift in the oxido-reduction equilibrium of the blood can be expected due to the strong activation of the macrophage proinflammatory response (Cole *et al.* 2008) and oxidative stress in the infected host. Cyclic voltammetry is suggested here as a tool for the estimation of free blood antioxidants. Biosensors have been found useful for the detection of antibodies against *F. tularensis* and the diagnosis of tularemia (Pohanka *et al.* 2007a) as well as for bacterial cell detection (Pohanka *et al.* 2008). Electrochemical assays were also found useful for the monitoring of e.g. flavonoids (Adam *et al.* 2007) and thiols (Supalkova *et al.* 2007).

The main purpose of the present study was to measure total low-molecular-weight antioxidants using cyclic voltammetry. A wild *F. tularensis* strain was used in order to compare the development of blood antioxidants following the experimental infection of BALB/c mice (*Mus musculus*), which represents a highly susceptible species, and the common vole (*Microtus arvalis*), a small mammalian host with a two-orders-of-magnitude lower susceptibility as confirmed by evaluating median survival, survival curves, LD50, minimum infectious dose and bacterial burden development (Bandouchova *et al.* 2009a). The present study dealt in a novel way with an as-of-yet undocumented pathological mechanism and infection progress.

MATERIAL AND METHODS

Experimental microorganisms. A wild strain of F. tularensis isolated from a European brown hare from South Moravian region (Czech Republic) in 2004 was used for the experimental infections in this study. A liver sample from the hare was mechanically homogenized and spread onto McLeod agar supplemented with Iso VitaleXTM (Becton-Dickinson, San Jose, CA) together with bovine hemoglobin. Plates were incubated under humid aerobic conditions for at least 24, but not more than 48 hours. The microorganism was typed as *F. tularensis* subsp. *holarctica* in a previous, thorough proteomic study (Pohanka *et al.* 2007b).

Preparation of Francisella tularensis for experimental infection. Experimental infections were performed

by a suspension of *F. tularensis* cells rinsed down from the culture growing on blood agar supplemented with cystine, using a sterile physiological saline solution. No adjuvant was added. After a thorough mixing we measured the turbidimetry of the suspension at 605nm of wavelength using a spectrophotometer (Unicam Helios Gamma&Delta, Spectronic Unicam, United Kingdom) in order to determine the number of bacterial cells per volume units according to Mc Farland´s standard (Murray *et al.* 2003). The obtained count was only approximate and served to estimate the dilution necessary to achieve the required dose. An exact infectious dose was then determined by plating 10-fold serial dilutions and by counting colony-forming units (CFU) in the suspension administered to the experimental animals. Colonies were counted after 72 h of incubation at 37° C.

Experimental animals. Laboratory mice (*Mus musculus*) and common voles (*Microtus arvalis*) were used for the study. BALB/c mice were purchased from a commercial breeder and introduced in the experiment at the age of 8 weeks with a body weight of 25 g. During the early spring, adult common voles were trapped in the wild as over-wintered animals. After checking their state of health (for ensuring a healthy appearance, an excellent nutritional state, freedom from tularemia based on agglutination tests) and sexing the specimens captured, pairs were formed and kept under laboratory conditions in enclosures for rodents. The resulting offspring were then introduced into the experiment at the age of two months with a body weight from 14 to 21 g. They were fed granules for rodents, mixture of seeds, meadow grass and hay and provided with drinking water *ad libitum*. Experiments were performed in compliance with laws for the protection of animals against cruelty and approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

Experimental design. Blood samples for the estimation of total blood antioxidants in laboratory mice and common voles were obtained following intraperitoneal infection with 160 CFU *pro toto*. With a total of 70 specimens from each species used in this experiment to evaluate the development of antioxidants in the course of tularemia, ten animals served as healthy controls and ten individuals were sampled every day post infection. Blood was collected by cardiac puncture using a 2 ml heparinized syringe, centrifuged immediately, and the plasma removed and frozen (-80 °C) until analysis by CV.

Histopathology was used for the control of infection progress. Tissue samples were collected to 10% buffered formalin, treated with a routine histological technique and embedded in paraffin. Sections of 5 μm were made of the paraffin blocks and stained with haematoxylin and eosin.

Cyclic voltammetry. The plasma total antioxidant capacity was estimated by cyclic voltammetry. The anodic current corresponds to the concentration of antioxidants, i.e. compounds that are able to donate electrons (Psotova *et al.* 2001; Chevion *et al.* 2000). The electrochemical device EmStat (PalmSens, Houten, Netherlands) and screen-printed strips with platinum working - 1 mm in diameter – silver/silver chloride reference and platinum auxiliary electrodes (AC1W2R2, BVT, Brno, Czech Republic) were used throughout experiments and connected to the device by a connector KA1.3. The strip was fixed horizontally and 20 μl of the plasma sample were spread over electrodes. The electrochemical current was registered in the selected potential intervals and the raw data was processed and stored by the software PSLite 1.7.3 (PalmSens, Houten, Netherlands). The scanning rate was adjusted up to 100 mV/s. We decided to evaluate the current at selected voltages in a similar way as used in references (Psotova *et al.* 2001; Zielinska *et al.* 2008).

Data processing. Experimental data were processed using PSLite 1.7.3 and Origin 6.1 (OiginLab Corporation, Northampton, MA, USA). Statistica for Windows 7.0 (StatSoft, Tulsa, OK, USA) was employed to evaluate the differences in total antioxidant levels among the groups (controls and samples taken on individual days post infection) using the Tukey multiple comparison test.

RESULTS AND DISCUSSION

Laboratory BALB/c mice and common voles, representing the murine and microtine rodent species, respectively, were used in the experiment to evaluate their susceptibility to the *F. tularensis* infection and to identify the differences in their antioxidant defense during the course of tularemia.

First, 7 groups of 10 specimens of experimental animals were intraperitoneally injected with 1.6×10^{4}, 1.6×10^{3}, 1.6×10^{2}, 1.6×10^{1}, 1.6, 1.6×10^{-1}, and 1.6×10^{-2} CFU. Then the animals were examined twice daily for developing signs of infection. Data on days (hours) elapsed from inoculation to death were then recorded in order to compute the median lethal dose (LD50), which was 1.1±0.5 CFU in mice and 18.2±0.2 CFU in common voles. Clinical symptoms of tularemia were the same in both rodent species and differences in mortality curves are shown in ***Figure 1***.

For further experiments, a dose of 160 CFU was chosen in order to avoid discrepancies in mortality and disease progress around the LD50 because a 100% mortality was expected with this infectious dose.

Cyclic voltammetry calibration. Plasma spiking with ascorbate and cysteine was chosen to estimate the sensitivity of the device used in the experiment. Cysteine was used as a representative of free thiol-bearing molecules oxidisable to the dithio form. Ascorbate was selected for another molecule participating as an antioxidant in the body. These two molecules were chosen as substances for the device testing, especially the electrochemical

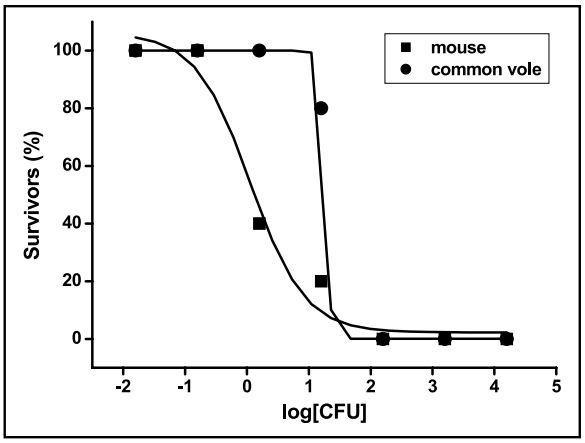

Figure 1. Mortality curves following intraperitoneal infection with seven serially (ten-fold) diluted doses of F. tularensis (colony forming units, CFU). Laboratory BALB/c mice (Mus musculus) and common voles (Microtus arvalis) were used throughout the experiment.

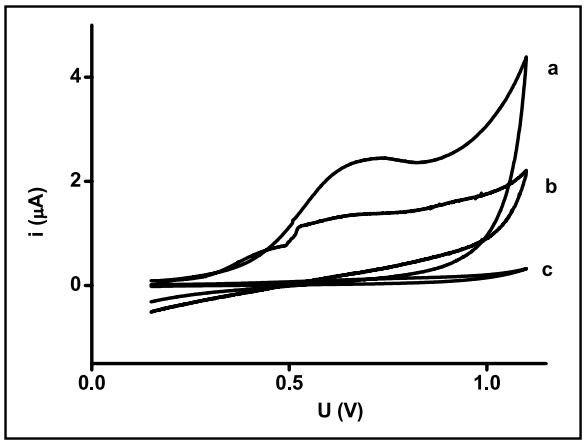

Figure 2. Examples of real voltammograms. (a): plasma from common vole after being infected with tularemia for five days; (b): plasma from healthy voles; (c): control measurement of phosphate buffered saline.

strip, and for verification of assay feasibility, but not for the description of the electrochemical oxidation of antioxidants that is well known (Psotova *et al.* 2001; Chevion *et al.* 2000) due to its previous comparison with more elaborative photometrical assays that showed a very good correlation (Khaw & Woodhouse, 1995). The antioxidative effect of ascorbate is based on keto – enol redox changes. When comparing voltammograms, a steady proportion between different concentrations of antioxidants dissolved into pooled mouse plasma samples was observed. The peak of the ascorbate was clearly visible at voltage of 658±17 mV. Cysteine was oxidized at the same voltage of 685±70 mV. The Faraday current was observed at approximately 950 mV, and then a strong and non-specific increase of the current in the region above 1 V followed. A good correlation was achieved between ascorbic acid and cysteine when both compounds were assayed in the concentration of 20 – 200 μM. The background signal of the control plasma was approximately 1800 nA. The addition of 200 μM of ascorbic acid as well as cysteine doubled the achieved

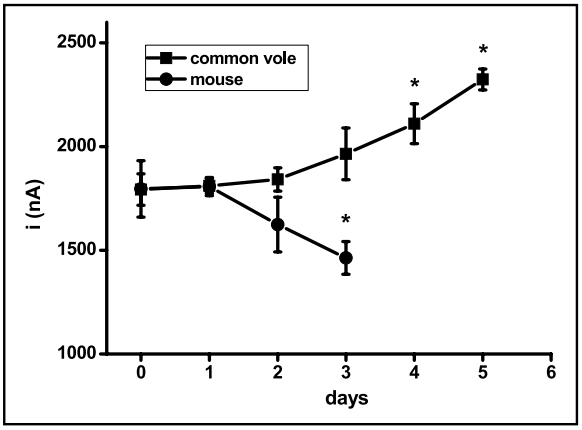

Figure 3. Total antioxidant levels expressed as the current provided by the antioxidant redox reaction during cyclic voltammetry in mice and common voles infected with F. tularensis. The current is measured at the peak or wave at 673 mV and plotted against the duration (days) of infection. Zero indicates data from control (i.e., healthy non-infected) specimens. Error bars express standard deviation (n=10). Asterisks indicate the significance against control group (day zero) at the probability level of 0.01.

current. Due to the good correlation with the linear model, we concluded that the signal provided by control plasma was caused only by the concentration of 200 μM of oxidisable antioxidants. Our results achieved for ascorbate corresponded with the expected data (Khaw & Woodhouse, 1995). The peaks observed when assaying real plasma samples were related to the potential ranging from 650 to 700 mV. Lower potentials relating to uric acid provided no specific changes. The peak in unspiked plasma samples was found at a potential of 673±35 mV. The current at 673 mV was considered when plasma samples provided no specific peak but a typical wave.

Cyclic voltammetry in plasma samples from F. tularensis infected animals. CV was employed to estimate the development of antioxidant levels in the course of tularemia in two small rodent species (the laboratory mouse and the common vole) of similar body weight, but having a different susceptibility to the *F. tularensis* infection. The advantage of CV is that it assays the overall redox values of the examined samples, which correspond to the sum of compounds having antioxidative properties. The calibration of the measuring system was made using ascorbate and cysteine. However, the number of chemical antioxidants occurring in the body is much more extensive (Salvi *et al.* 2002). Examples of voltammograms measured by CV are shown in ***Figure 2***. Here, a gradual increase of the detected current in the positive voltage is obvious.

The data obtained by CV from plasma samples of laboratory mice and common voles was transformed into graphs presented in ***Figure 3***. Two principally different dependencies were found. Laboratory mice responded to the administration of *F. tularensis* and the development of tularemia by exhibiting a significant decrease

in plasma antioxidant levels. The correlation between the achieved Faraday current vs. infection progress pointed to a strong decrease of low-molecular-weight antioxidants in mice suffering from tularemia. However, in the infected voles, the low-molecular-weight antioxidants were gradually increasing. Interestingly, both species in the experiment were infected with a dose of *F. tularensis* high above LD50, so this development was expected. The administered dose resulted in a fatal outcome in both experimental species. However, common voles survived for five days, and the mice only three days. The rapid depletion of total antioxidants noted in the BALB/c mice in the course of tularemia is another demonstration of their higher susceptibility to the infection compared with the common vole, which was able to respond to the developing infection with a gradual increase in antioxidant capacities (cf. Figure 3). These findings confirm the expectation placed on low-molecular-weight antioxidants to combat diseases or minimize the effects of high-level toxicity from various sources (Bauerova *et al.* 2008; Ujhazy *et al.* 2008).

These results are in agreement with our previous findings that compare the susceptibility of selected murine and microtine reservoirs to infection with a wild-strain *F. tularensis* subsp. *holarctica* (Bandouchova *et al.* 2009 a,b). Generally, tissue bacterial burdens in common voles start to develop after exposure and amount to lower levels than in laboratory mice. The approximate tissue and blood burden of *F. tularensis* cells reached 10^4 CFU within two days in mice infected with a dose of 160 CFU, while the same infectious dose in common voles lead to the burden of 104 within nearly four days. Therefore, the higher and more quickly developing tissue and blood bacterial burdens of *F. tularensis* in mice result in higher levels of oxidative stress induced by the bacterium, therefore reducing the host-defence mechanisms' ability to mobilize the energy needed to combat the infection (Van Amersfoort *et al.* 2003). Hypertriglyceridemia is known to occur as a response to bacterial endotoxin, and triglyceride-rich lipoproteins are thought to be agents of the innate immunity of the host. However, it seems that the antioxidant defence is quickly overcome by the infection in mice despite energy mobilization. A similar finding, i.e. that genes that lead to the depletion of glutathione are upregulated following infection by *F. tularensis* LVS, has already been made in vitro using the mouse macrophage cell line J774 (Andersson *et al.* 2006). However, the increase of the vole's antioxidant capacity may be responsible for its lower susceptibility to the infection with *F. tularensis*. The correlation between antioxidant levels and disease progress is well known. For example, antioxidant response is recognized in cancer patients (Mantovani *et al.* 2002) using previously optimized measuring protocols (Chevion *et al.* 1999) and also in FIV infections in cats (Webb *et al.* 2008).

The progress of infection was also evaluated using histopathology. In *Microtus arvalis* we observed mild to severe interstitial pneumonia, diffuse necroses in the spleen, focal necroses in the liver and moderate vacuolization of hepatocytes. The main findings in BALB/c mice were severe necroses in the spleen and mild focal necroses in the liver. Tissue changes correlate well with findings of levels of low-molecular-weight antioxidants. In mice it seems that the lower the concentration of antioxidants, the more severe changes occur in the tissues, especially in the liver. The same extent of tissue damage was found at least two days later in post infection in the voles when compared with mice.

The evaluation of antioxidant levels may be used as a biomarker of the disease's progress. However, as only the non-enzymatic antioxidant defence component has been examined in the present study on tularemia in BALB/c mice and common voles, changes in both the enzymatic and low-molecular-weight antioxidant defenses should be evaluated in future experiments in more extensive and elaborate research projects.

CONCLUSIONS

In this present study, cyclic voltammetry has been found to be a simple and feasible method for measuring blood antioxidant levels in *Francisella tularensis*-infected hosts. The infection from F. tularensis is known to induce an oxidative stress upon the host. The correlation between oxidative status and infection progression was estimated. Interestingly, BALB/c mice and common voles differed significantly in the pattern of antioxidant response, which might explain the differences in their susceptibility to infection. It seems that the antioxidant defence capacity becomes rapidly depleted by the infection in mice. However, the increase of antioxidant capacity in the common vole may be responsible for its lower susceptibility to the infection from *F. tularensis* and therefore, its longer survival. As the calibration of the cyclic voltammetry measuring device included cysteine, representing thiol antioxidants and ascorbate (non-enzymatic blood antioxidants only), studies concerning the enzymatic component of the antioxidant defence system are needed to further our knowledge of the pathogenesis of this complex zoonotic disease.

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