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Microbiological and Clinical Aspects of Tularemia Disease

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Summary

Francisella tularensis ranks among the most significant biological agents exploitable for bioterrorism and biological warfare. The reasons why *F. tularensis* is considered to be a militarily exploitable organism with high relevance for human as well as veterinary medicine are high virulence, quick spreading through vectors and stability in the aerosol combined with good pulmonary transmission. The aim of the text is to summarise in a transparent way current knowledge of tularemia and outline the destiny of the generator of the disease in the organism. Attention is paid to immunological aspects of the disease, ways of treatment and diagnostic processes.

Keywords: *Francisella tularensis*; Tularemia; Immune system; Scavenger cells; TLR receptors; Antibodies; Antibiotics; PCR

Introduction

Francisella tularensis (*F. tularensis*) is a facultative intracellular gram-negative bacterium. Morphologically it is an aerobic, non-motile coccobacillus which does not have the capability of creating spores and whose dimension does not exceed 0.8 μm. *F. tularensis* is the generator of the zoonotic disease of tularemia. The discovery of the generator of the disease dates to the beginning of the twentieth century when a strange plague of rodents spread in the area of Tulare in California. The generator was described in the year 1911 [1] and isolated a year later [2]. At present, the population of hares, rodents and other small mammals in wide open space is considered as the significant reservoir of tularemia. Among significant natural reservoirs, e.g. muskrats *(Ondatra zibethicus)*, beavers *(Castor canadensis)*, water-rats *(Arvicola terrestris)*, hares *(Lepus spp)* and field mice *(Microtus arvalis)* can be rated [3]. The vectors are mainly ticks, but mosquitos have also been speculated on [4,5].

A culture of *Francisella tularensis* LVS on McLeod´s chocolate agar

F. tularensis ranks among the most significant biological agents exploitable for bioterrorism and biological warfare [6]. The reasons why *F. tularensis* is considered as militarily exploitable organism are high virulence, quick spreading through vectors and stability in the aerosol combined with good transmission by respiratory way [7] (Figure 1).

The aim of this text is to summarise in a transparent way current knowledge of tularemia and outline the life cycle of the generator of the disease in the organism. Attention is paid to immunological aspects of the disease, ways of treatment and diagnostic processes.

The prevalence of affection by tularemia in people in the Czech Republic was of 0.5 % in the year 2010, a year earlier, 0.62 % for 100 000 inhabitants (SZÚ, EPIDAT). The most represented reservoir animal are mice, hamsters and hares (primarily Russia), from vectors consequently mosquitos (Sweden, Finland, Russia) and ticks (Central Europe) [8]. It results from the study carried out in the Republic of Kosovo that was done in the period of May – July the presence of *F. tularensis* in ticks was of 65 %, the presence at reservoir animals, above all at hares, was in the period of November – December of 33 %, in the period of May – July of 22 % [9].

F. tularensis as generator of tularemia

We distinguish in total four subspecies: *F. tularensis* subspecies (subsp.) *tularensis* (or also *F. tularensis* type A), *holarctica* (*F. tularensis* type B), *novicida* and *mediaasiatica*. *F. tularensis* occurs in the United States, Canada, countries of Europe, Asia and in Japan. The most virulent tribes, belonging to the *tularensis* subtype, range in the territory of North America. In Europe and Asia, there occurs predominantly the *holarctica* subtype, which is found also in the territory of the Czech Republic. *F. tularensis* is the generator of tularemia. The transmission to man can occur in various ways, according to which we distinguish in total four forms of disease: ulceroglandular, oculoglandular, typhoid and pulmonary. For the ulceroglandular form of disease, the ulcerative lesion in the place of penetration through skin and enlargement of declining lymphatic nodules is characteristic. In the oculoglandular form, the infection penetrates through conjunctiva, it manifests by blenophthalmia. The typhoid form is accompanied by haemorrhage and ulcerous alterations in the alimentary tract, it happens by ingestion of contaminated food or water. The most serious is the pulmonary form which originates either by direct inhalation of contaminated

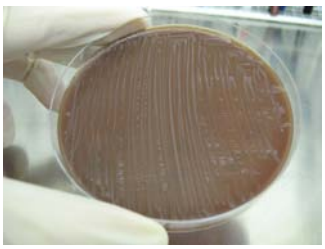

Figure 1: A culture of Francisella tularensis LVS on McLeod´s chocolate agar.

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aerosol or as a result of haematogenous dissemination of bacteria from distal body parts. It manifests as a systemic disease with these manifestations: pharyngitis, trachitis or bronchitis, pulmonitis, pleuritis and incrassation of nodules, which can lead up to pneumonia with respiratory failure and consequent death.

The disease manifests clinically after 3 to 7 days of incubation. Patients suffer from the feeling of a chill or shivering fit, temperature rises to 38-39°C or even higher. Vehement pains of head, muscles and joints, vertigo, general asthenia, loss of appetite, insomnia and night sweats are described.

Diseases are caused in people by *F. tularensis* subsp. *tularensis* and subsp. *holarctica*, whereas *F. tularensis* subsp. *holarctica* is responsible for less serious cases of tularemia. The LVS (Live Vaccine Strain) vaccine tribe – still used for the study of tularemia, was deduced just from this subtype. The *novicida* and *mediaasiatica* subtypes are non-pathogenic for human beings [10].

Pathogen invasion

In the organism, *F. tularensis* is found in scavenger cells, dendritic cells, hepatocytes, alveolar epicytes and extra-cellular then in the blood of infected animals. The virulence of this bacterium is connected with its ability of replication inside host cells. It escapes quickly from phagosome and replicates in cytosol of host cells. After replication (c. 24 hours after infection) it enters the endosome part of a cell [11].

F. tularensis is growth-demanding, it requires the presence of iron and compounds containing –SH group (for instance, cysteine which is an important growth factor for it). A study with ggt mutant of *F. tularensis*, which is not able to split gamma-glutamyl of cysteine bond and obtain thus cysteine by splitting of glutathione, was made. The *in vivo* experience with this mutant on mice BALB/c demonstrated a difference in medium fatal dose by the LVS tribe (10^{1}) and ggt mutant ($10^{4.8}$) [12].

The bacterium is, upon entering the scavenger cell, surrounded by long, non-symmetric loops of pseudopodia and ignominiously pulled in the cell. This way of ingestion is different against other bacteria which enter the cells by standard phagocytosis during which pseudopodia of cytoplasm are smaller and symmetric. An accurate, by a receptor mediated mechanism of entering into the cell is not known, but we suppose involvement of complement receptors, Fc – receptors, a mannose receptor and TLR (Toll-Like Receptor) receptors, specifically TLR2. We can result from the knowledge of capability of scavenger cells to recognize structures at the surface of microorganisms. There are concerned groups of molecules whose structure is typical for individual microorganisms, e.g. endotoxins of gram-negative bacteria, peptidoglycans, glucans, phospholipides, etc., which we include under the name of PAMP structure (Pathogen Associated Molecular Patterns). These molecules are recognized by the so-called PRR (pathogen recognition receptors) which are expressed predominantly at cells presenting antigen (scavenger cells and dendritic cells). The main task of these receptors is opsonization, phagocytosis, activation of complement and pro-inflammatory signal ways and induction of apoptosis. As has already been noted, the mannose receptor, scavenger receptor, complement receptor, Fc – receptor and the TLR group (Toll – Like Receptors) of receptors ensure the phagocytosis of microorganisms on the surface of scavenger cells. The name TLR is derived from the receptor described at fruit fly *(Drosophila)*. At present, thirteen TLRs are known [13].

Activation of immune system

After activation of different types of TLR, there occurs the initiation of a signalling cascade whose character depends on the quantity of lipopolysaccharide (LPS) antigen and three-dimensional shape of its end point, constituted by lipid A [14].

TLR receptors are consequently primarily expressed on cells presenting antigen. The recognition of microbial products by these receptors leads to the activation of nuclear factor kappa B (NF-κB), mitogen-activated protein kinase and to induction of co-stimulating molecules. The ability of TLR receptors to influence the production of immune-regulation cytokines and modulation of expression of co-stimulating molecules reflects their decisive role at protal as well as adaptive part of immune system which we can illustrate in the example of TLR-2 receptor. By means of this receptor, *F. tularensis* stimulates the production of pro-inflammatory cytokine TNF-α and increased regulation of expression MHC class II, CD80, CD86 and CD40 [15].

TLR receptors are not, however, only a domain of cells presenting antigen but also of mature B cell expressing these receptors (TLR-4 and TLR-2) on its surface. During recognition of microbial products, there occurs activation of other cells, above all of scavenger cells, by means of cytokines IL-6, TNF-α and β and IL-12. B cells can be activated by signalization through TLR-4-MyD88 path. This way of B cell activation was monitored with the help of the effect of thermal shock protein of the size of 60 kDa (HSP60) in mice which have never been presented to any pathogen so far. The soluble HSP60 induced proliferation of B cells and secretion of IL-10 and IL-6. B cells also regulated increasingly the expression of MHC II and several significant surface markers (CD69, CD40 and B7-2) [16].

Another way of B cell activation is by the stimulation of lipopolysaccharide (LPS), which leads to proliferation and secretion of IgM. B cells have two TLR receptors which can mediate LPS signalization, TLR-4 and RP105 (CD180). RP105 is a trans-membrane protein of type I of the size of 105 kDa. Its protective function, protecting B cells against apoptosis induced by irradiation, is stated. RP105 is expressed predominantly on mature B cells at mice and on human dendritic and B cells [17].

Antibiotics treatment

In previous years, aminoglycoside antibiotics were used for the treatment of tularemia, above all streptomycin; dose of 7.5-10 mg/kg every 12 hours *i. m.* for the duration of 7-14 days. Streptomycin is presently used for its serious adverse effects, such as ototoxicity, only in isolated cases (e.g. at tularemic meningitis). It was substituted by another aminoglycoside antibiotic – gentamicin, which is administered in a dose of 3-5 mg/kg/day in intravenous infusion also for the period of 7-14 days. It is recommended to administer simultaneously doxycycline of 100-200 mg once a day *p. o.* [18].

A study by Scandinavian microbiologists proves that another potential alternative for the treatment of tularemic infection are quinolone antibiotics, e.g. ciprofloxacin, levofloxacin, grepafloxacin and trovafloxacin. A Minimum Inhibitive Concentration (MIC) of representatives of several classes of Antibiotics (ATB) in 38 isolates, from that 20 of human origin and 18 of animal origin (16 samples from a hare and 2 samples from a muskrat) was set. In the experiment, collection tribes ATCC 6223 *F. tularensis* subsp. *tularensis* and ATCC 29864 **F. tularensis** (biovar **F. tularensis palaearctica**) were used as a control. Quinolone ATB demonstrated MIC_{90} smaller than 0.05 mg/L;

in comparison with streptomycin (4 mg/L) and gentamicin (1 mg/L) [19].

Diagnosis of tularemia

Tularemia can be simply diagnosed by assessment of specific antibodies level in serum or plasma. The increased level of antibodies can be diagnosed even a few days after infection as evidenced for BALB/c mice infected with *F. tularensis* LVS in an experiment [20]. Though the immunodiagnosis is quite sensitive, it can suffer from low specificity as cross reactivity between antibodies against *F. tularensis* and *Brucella* sp. may be expected. For this reason, brucellosis cannot be revealed properly until tularemia is not excluded [21]. The cross reactivity of antibodies can be reduced by addition of dithiothreitol into the examined sample [22].

In the body, bacterial burden culminates approximately five days after disease beginning and it vanishes after approximately 20 days [23]. The presence of bacterium in host can be revealed by Polymerase Chain Reaction (PCR) with good sensitivity and selectivity. Proves of tul4 and fopA genes coding 17 and 43 kDa proteins of *F. tularensis* outer membrane are suitable for tularemia diagnosis [24,25]. Amplification of genes for chaperones cpn10, cpn60 and 16S rRNA is known and recommended by some investigators for a reliable diagnosis of tularemia [26,27]. Biopsies of lymph nodes and plasma are samples well suitable for the assays purposes.

The genetic investigation is reliable for reveal of running tularemia. On the other hand, resolving of the disease leads typically to negative prove of the disease by genetic tests. In an example, twenty-year old woman was infected with tularemia five months prior to laboratory examination [28]. Specific antibodies as well as C reactive protein were significantly elevated; however, genetic tests based on PCR were negative. Considering actual methods for tularemia diagnosis, PCR (mainly in real time variant), microagglutination, immunofluorescence and enzyme linked immuno-sorbent assay can be mentioned as the main tools for a reliable reveal of tularemia [29].

Necessity to diagnose tularemia is conditioned by epidemiologic situation. Presence of tularemia in wild animals as a natural reservoir fluctuates in course of weather conditions and social aspects [30]. Tularemia is a zoonotic disease and humans can be simply infected from animals. The most probable is contact with animals in lower altitude especially in alluvial regions [31]. Gradation of rodents populations are a risk factor for tularemia outbreak [32-34] therefore the laboratory diagnosis should be done for patients with an infectious disease coming into contact with wild animals.

Conclusion

Tularemia is a dangerous infectious zoonotic disease which can be brought in the population from animal reservoirs. In spite of a low contamination of the European population, tularemia cannot be considered as well manageable infectious disease. On the contrary, its treatment and diagnostics are relatively demanding and are connected to specifics related to the pathogenesis of microorganisms. This text summarizes in a transparent form the necessary data for understanding pathological processes of tularemia, its diagnostics and pharmacological treatment and submits them to the reader compact with references to specialized works dedicated to real aspects.

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One of the goals of this project is the testing of the antibiotic properties of newly synthesized substances on a series of selected microorganisms, specifically on *Bordetella pertussis, Bacillus anthracis, Yersinia pestis*, *Escherichia coli*, *Bacillus subtilis*, *Listeria monocytogenes*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Mycobacterium tuberculosis*, *and Staphylococcus aureus* and on the above mentioned *Francisella tularensis*.

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