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II. POSTER PRESENTATION

TEMPERATURE SENSITIVE LESIONS IN VALAB AFFECT LIPOPOLYSACCHARIDE ASSEMBLY AND DEOXYCHOLATE SENSITIVITY AT THE NON-PERMISSIVE TEMPERATURE

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Introduction: We have recently discovered agenetic locus in Francisella novicida that contains an apparent operon consisting of two open reading frames, valAB. F. novicida strains thought to harbor mutations in valAB are defective for growth in macrophages and exhibit increased sensitivity to serum and/or deoxycholate, thus suggesting compromised outer membrane integrity. These two genes show high identity at the deduced amino acid level to the E. coli genes m sbA and orfE, respectively. Both ValA and MsbA are members of the superfamily of ATP binding cassette (ABC) transporters. The purpose of this study was to investigate the hypothesis that ValAB is involved in transport of lipopolysaccharide (LPS) to the outer membrane.

Materials and Methods: We constructed conditional lethal mutations in valAB. To provide the proper genetic background we constructed an E. coli W3 110 strain harboring a temperature sensitive plasmid encoding the m sbA locus, a chromosomal copy af the m sbA locus interrupted by a Cm^R cassette and a recA::Tn10 locus. This strain was named MKM2. In a separate strain, valAB was subcioned and mutagenized with nitrosoguanidine, and subsequently transformed into strain MKM2. 1000 transformants were picked to duplicate agar plates that were incubated at 30°C and 42°C. A plasmid named pMKM3 from one of these temperature sensitive strains was chosen for further study. pMKM3 was transformed into W3110 and the Cm-interrupted m sbA locus was introduced into the chromosome by P1 transduction. The resulting strain, MKM55, was unable to grow on agar plates at 42°C. A control strain (MKM50) harboring a wild type copy of valAB continued growth at 42°C. The location of the temperature sensitive mutations in valAB was determined by DNA sequence analysis. To test the hypothesis that valAB may be involved in LPS transport we introduced a clone of gseA into strains MKM55 and MKM50. The Chlamydia trachomatis gene gseA encodes a Kdo transferase that produces aChlamydia-specific LPS epitope. This epitope is easily detectable in recombinants eith a monoclonal antibody. Thus, strains MKM5523 and MKM5023 harbor a defective m sbA/orfE locus, an IPTG-inducible subclone of gseA, and either a temperature sensitive or wild type allele of valAB, respecively. The IPTG-induced expression of the Chlamydia LPS epitope by both strains was determined at the restrictive and permissive temperatures by immunofluorescense microscopy.

Results: Sequencing of the temperature sensiive valAB locus located two single amino acid substitutions in the valA coding sequence, consisting of a S to F change at position 453, and a T to I change at position 458. Strain MKM55 grown at 42°C exhibited 100-fold greater sensitivity to 5% deoxycholate as compared to control strain MKM50. Immunofluorescence microscopy studies demonstrated that strain MKM5523 exhibited a 10-fold decrease in expression of the Chlamydia LPS epitope upon a shiff to 42°C as compared to the control strain MKM5023 at the non-permissive temperature.

Conclusions: Here we demonstrate that valAB can suppress the lethal phenotype of a msbA/orfE mutation in E. coli. Furthermore, through the use of a temperature sensitive allele of valAB, we show that E. coli cells defective in valA and msbA-/orfE exhibit increased sensitivity to deoxycholate, and impaired cell surface expression of the LPS epitope synthesized by GseA, a Chlamydia trachomatis Kdo transferase. Thus, we hypothesize that valAB is involved in transport of LPS to the outer membrane.

RESTRICTION MAP OF pFNL10 PLASMID

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Introduction: pFNL10 plasmid has been described, and its restriction map for Bg1II, XbaI, EcoRY, HindIII endonucleases has been built. PFNL10 is capable of being replicated and stably inherited in F. tularensis (1). Up to the present day functional role of pFNL10 plasmid in Francisella spp. has not been elucidated. Vectors for cloning heterologous genes in F. tularensis were constructed on the ba-se of pFNL10 plasmid (2,3).

Endonuclease sites are located in the plasmid irregularly. In the present study additional restrictases were used for the building of a detailed restriction map of pFNL10 in order to perform nucleotide sequence and genetic analysis.

Materials and Methods: F. tularensis 15/10, F. tularensis 15/10 (pFNL10) (collection of SRCAM) were used.

Cultivation of F. tularensis, plasmid DNA isolation, gel electrophoresis of DNA, plasmid restriction, ligation were carried out as described in (2).

Plasmid DNA pSa was kindly submitted by Dr. A. Pomerantsev.

Electroporation of F. tularensis was performed at a concentration of IO^{10} cells per 1 ml, 2.5 kV and pulse duration of 2.5 msec., distance between the electrodes being of 2 mm. 0.5 M sucrose,

O.OO1mM EDTA, pH 7.5, was used as a buffer. The culture grown on antibiotic-free agar at 37°C for 2 h was inoculated on a selective agar and incubated at 37°C for 4 days.

Results: Endonuclease recognition sites for C1aI (1 site), DraII (1 site), SphI (1 site), HincII (2 sites), SspI (5 sites) were localized. Restriction map for the restrictases mentioned was built. It was found that recognition sites for AsuII, AvaI, AvaIII, Bc1I, BspMII, Eco47III, HpaI, KpnI, MluI, MspI, NdeI, SnaI, SnaBI are absent. T4 phage DNA ligase treatment of a mixture of pFNL10 plasmid HindIII-fragments and plasmid pSa HindIII-(3.1 kb) fragment with cat-gene, transformation of it into F. tularensis 15/10 cells followed by the selection of the transformants on a chloramphenicol- (3 mg/ml) containing medium resulted to the clones with plasmids of one and the same size. PFNL1010 plasmid was found to consist of three HindIII-fragments: 3.1 kb,1240 bp and 670 bp. Restriction map of plasmid pFNL 1010 containing a region required for pFNL 10 plasmid replication, for endo-nucleases: SspI, DraI, MboII, VspI was built.

Conclusions: Restriction sites for endonucleases ClaI, DraII, SphI, HincII, SspI were localized on plasmid pFNL10. PFNL10 plasmid region required for plasmid replication in F. tularensis was determined.

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REC A -LIKE *FRANCLSELLA TULARENSIS* STRAIN: ISOLATION AND PROPERTIES

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Introduction: Development of live complex vaccines proposes elaboration of recipient strains permitting stable segregation and structural inheritance of heterological genes. Isolation of recA mutants may be one of the ways in achieving of this aim. It may be supposed that the characteristic feature of recA mutants will be the low residual virulence due to their inability for synthesis of stress proteins in *vivo*. This supposition comes out from the biological role of recA system in activation of SOS answer by proteolysis of Lex A repressor. Isolation of recA - like mutants of vaccine strain *of F.tularensis* was the aim of the presented study. This microorganism and especially its recA -like mutants may be a prominent candidate vector for delivery of heterological protective antigens against pathogens with intracellular localization.

Materials and Methods: Next strains were used: *E.coli* HB101 rec, *F.tularensis* vaccine strain 15/10 from culture collection of the State Research Centre of Applied Micribiology. Cultivation *of E.coli* and *F.tularensis* cells was carried out using media (Gerhard P., 1982). Media were solidified if necessary by 1,5% agar-agar. *E.coli* and *F.tularensis* cells were electroporated in buffer containing 0,5 M sucrose, 1 mM EDTA, pH7,5, with 12,5 kV/cm electric pulse of 2.5 msec. *F.tularensis* recA-like gene was cloned in such a way: BamHI fragments *of F.tularensis* DNA were ligated in pBR322 plasmid, *E.coli* cells were transformed and clones were selected using media with mitomycin C (0,5 mkg-/ml). *F.tularensis* mutants were isolated as it is described below. Suspension *of F.tularensis* cells containing 10^8 CFU/ml was UV irradiated in dose providing 1-5% of survival. The irradiated cells grown for 18 hours on solid media, then the culture was collected and inoculated on media without selective agent, and after that growth control of separate colonies was carried out using the media with mitomycin C (0,5 mkg/ml. Mutants with lost resistance to this antibiotic were isolated. Mutation complementation in rec-like gene *in F.tularensis* mutant cells having reduced resistance to UV irradiation and mitomycin C was carried out by electroporating of pHVT1 plasmid into the cells. PHVT1 plasmid contained recA - like gene of F. *tularensis* and was stabily replicated in this microorganism. Residual virulence and persistent activity of recA - like vaccine *F.tularensis* mutants were evaluated on mice after subcutaneous inoculation of 10^3 - 10^5 cells.

Results: Cloning *of F.tularensis* recA - like gene in pBR322 plasmid provided *E.coli* HB101 (pRU5) clones with enhanced resistance to UV irradiation and mitomycin *C. F.tularensis* fragment DNA (6.3 kb) from pRU5 plasmid was considered to be the rec A -like gene because of its restriction profile identity to earlier published one [Berg et al, 1992]. Mutant *F.tularensis* clones with enhanced sensitivity to UV irradiation and mitomycin C were isolated as the result of selection of UV - irradiated cells. Electroporation of such mutant *F. tularensis* cells with pHVT I plasmid restored the resistance of the mutants to above mentioned agents, up to the level *of F.tularensis* 15/10. Persistent properties of recA -like mutant *F.tularensis* cells in mice were the same as *F.tularensis* 15/10 ones. The bacteria were isolated from the spleen and lymphatic nodes.

Conclusions: 1. RecA -like mutant variant of vaccine *F.tularensis* strain was isolated by means of UV irradiation and selection using media with mitomycin C.

2. Plasmid pHVT I containing 6.3 *kb F.tularensis* DNA fragment with recA-like gene complements

resistance to UV irradiation and mitomycin C in the mutant cells up to the level *of F.tularensis* 15/10.

3. Preserved persistence of vaccine recA-like mutant *of F.tularensis* strain provides a good opportunity for using it as a bacterial vector.

CHARACTERIZATION OF THE GROE OPERON ENCODING HEAT SHOCK PROTEINS CHAPERON-60 AND -10 *OF FRANCISELLA TULARENSIS* AND T-CELL RESPONSE TO THE PROTEINS IN IMMUNE INDIVIDUALS

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Introduction: Tularemia results in the induction of protective immunity in the mammalian host. The development of an effective host resistance is paralleled by the appearance of F. tularensis-induced immunospecific T cells. Besides the membrane proteins, there is little knowledge on T-cell stimulating antigens of F. *tularensis.* Among putative antigens are cytoplasmic stress proteins such as the heat shock proteins. In the present study the groE operon *of Francisella tularensis* LVS, encoding the heat shock proteins chaperon-10 (Ftcpn10) and Ft-cpn60, was sequenced and characterized and the T-cell response of LVS vaccinated individuals to Ft-cpn10, -60, and the third major heat shock protein Ft-DnaK was assayed.

Material and Methods: The *cpnl0* and *cpnó0* genes were amplified by the polymerase chain reaction using degenerate oligonucleotides derived from the N-terminal sequence of the two proteins and sequenced in both directions by the chain-termination method with the AmpliCycle sequencing kit (Perkin-Elmer, Norwalk, Cohn). The operon was further characterized by Northern blotting and Primer extension analysis. After separation of lysates of the LVS organism by two-dimensional gel electrophoresis, Ft-DnaK, Ft-cpn60 and Ft-cpn 10 were extracted and used as antigens in T-cell tests.

Results: The sequence analysis revealed the expected two open reading frames, encoding proteins of estimated M_r of 10,300 and 57,400. The deduced amino acid sequences closely resembled cpn 10 and cpn60 proteins of other prokaryotes. The genes constituted a bicistronic operon, the *cpn10* gene preceding the *cpn60* gene. Upstream of the *cpn10* gene, an inverted repeat and motifs similar to -35 and -10 sequences of σ^{70} -dependent but not of σ^{32} -dependent promotors *of Escherichia coli* were found. The inverted repeat of the operon resembled so called hairpin loops identified in other characterized prokaryotic groE operons lacking σ^{32} -dependent promoters. Primer extension analysis disclosed one and the same transcription start, irrespective of the presence or absence of heat or oxidative stress. When compared to nonprimed individuals, T cells from individuals previously vaccinated with live F. *tularensis* LVS showed an increased proliferative response to Ft-DnaK and Ftcpn60 but not to Ft-cpn10.

Preliminary results from ongoing studies also shows that after natural infection immune individuals react with a proliferative response to the heat shock proteins up to 30 years later. This reaction seems to be of Th1 type with production of INFγ in cell cultures, and both CD4+ and CD8+ cells respond.

Conclusions: Similar to the membrane proteins of F. *tularensis,* the heat shock proteins (Ft-DnaK, Ft-cpn60 and Ft-cpn10) seem to be T-cell reactive without being immundominant. In contrast to the regulation of the groE genes *in E.coli* the groE operon *of F.tularensis* is regulated without a σ^{32} -dependent promoter in accordance with that of certain gram-positive bacteria, purple bacteria and cyanobacteria.

PECULIARITIES OF RECOMBINANT F. TULARENSIS STRAIN R1A

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Studies on model bacterial recombinant strains with modified cell wall could clarify the role of individual cell structures of microorganisms in the pathogenesis of infections. Characterization of F. tularensis recombinant strain R1A is the objective of the present study.

F. tularensis strain 15, 15 R-form, recombinant strain RIA obtained by transferring genes responsible for F.tularensis subsp. tularensis B 399 A-Cole virulence factor into the recipient cells of R-form, virulent F.tularensis subsp.tularensis strain B399 A-Cole, were used. Microbiological, biochemical, immunochemical studies were performed by standard methods. Antibiotic resistance was studied by using solid media. Biological activity of strains was determined in experimental animals.

Recombinant strain R1A is a typical representative of F.tularensis as regards cultural, morphological and tinctorial properties. Recombinant strain RIA is referred to F.tularensis subsp. holarctica biovar II Ery^R. It lacks ability to digest glycerol, to exhibit citrullin-ureidase activity and shows resistance to erythromycin (Ery^R) and oleandomycin

(Olm^R). Cells of RIA are highly sensitive to the bactericidal effect of normal blood sera of sheep and rabbits and capable to grow at high temperature (+42°C) similar to virulent F.tularensis strains. R1A strain exhibits high antigenic activity. Subcutaneous administration in single dose of cell suspension results in rapid rise of specific antibodies titers as well as in slow and immediate type hypersensitivity reactions in guinea pigs and rabbits. Recombinant strain RIA is highly virulent for white mice, slightly virulent for guinea pigs and avirulent for rabbits. The strain partly protects guinea pigs from 100 DCL of highly virulent F. tularensis subsp. tularensis B 399 A-Cole.

The possibility of using F.tularensis R-form as a versatile bacterial recipient for gene cloning was first demonstrated. Recombinant microorganisms constructed seem to be a promising base for the development of live vaccines and diagnostic preparations. The strain obtained can be used as a model in the studies on the role of individual cell structures in the pathogenesis of infectious diseases.

FRANCISELLA TULARENSIS R-FORM A VERSATILE RECIPIENT FOR GENE CLONING

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Search of versatile recipients for the cloning of genes responsible for the virulence of different pathogens still remains to be an urgent problem. *F. tularensis* vaccine strain 15 (R-form) is proposed as a novel recipient. Model recombinant strains of different pathogenic Gram-negative microorganisms with acquired virulens on the base of the R-form were constructed and characterized.

F. tularensis vaccine strain 15, *F. tularensis* 15 R-form and recombinant strains constructed by transferring genes responsible for the virulence factors *of M.bovis* (BCG), strains RB7 and RB26; *P.pseudomallei* (C-141), strains RN4 and RM2; *P.mallei* (C-5), strain RC117; *F.tularensis (B* 399 A *Cole;* Schu), strains R1A and R5S, respectively, as well as phages T4 E. *coli* and MTPH2 *M. tuberculosis* were used. Vector construction and DNA transformation into the microbial cells were performed according to the methods previously developed (Patent N 96104090). Microbiological, biochemical, immunochemical studies were performed by standard methods. Antibiotic resistance was studied on solid media. Biological activity of strains was determined in experimental animals.

Two groups of recombinant strains on the base of R-form were constructed and characterized. The first group includes strains R1A, R5S, RN4, where virulence genes transferred into the chromosomes *of F.tularensis* R-form provide cell virulence and stably inherit the properties acquired. The second group involves strains RC 117, RM2, RB7, RB26. The virulence genes of these microorganisms are transferred into the vector plasmids. The strains lose their virulence when being reinoculated on nutrient media, and acquire it once more after passaging through susceptable animals. The both groups of recombinant strains showed high antigenic activity upon immunization of rabbits. Antigenic activity of RB7 and RB26 strains were found to differ from other microorganisms studied.

The possibility to use *F. tularensis* R-form as a versatile bacterial recipient for gene cloning was first demonstrated. Recombinant microorganisms constructed seem to be promising base for the development of live vaccine and diagnostic preparations. The strains obtained can be used as models in the studies on the role of individual cell structures in the pathogenesis of infectious diseases.

ROLE OF MOLECULAR CHAPERONES IN EVASION/SUBVERSION OF IMMUNE RESPONSE BY *YERSINIA PESTIS* AND *FRANCISELLA TULARENSIS*

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Introduction: Understanding the interaction of Y. *pestis* and F *tularensis* with the key components of the immune system is important for elucidation of the pathogenesis of bubonic plague and tularemia as well as other gramnegative bacterial infections.

Materials and Methods: The capsular f1 and pH6 operons of Y. *pestis* were cloned in E. *coli* and sequenced. The recombinant strains overproducing Caf1, Caf1M and pH6 (PsaA) proteins were designed. The proteins were purified to homogeneity by ion-exchange and gel-filtration chromatographies, and analysed by SDS-PAGE, circular dichroism (CD), difference adiabatic scanning microcalori-metry (DASM), spectrofluorimetry, amino acid analysis and different binding assays. The three-dimensional models of the proteins under analysis were constructed using the molecular modelling software packages SYBYL, QUANTA and Chem-X.

Results: We found that the capsular Caf1 subunit of Y. *pestis* shares homology with hIL-1, and its expression is mediated by the Caf1M protein which shares homology with a superfamily of immunoglobulin-like molecular chaperones. We discovered that Caf1M is the most typical

representative of the family characterised by an accessory sequence between F1 and G1 β-strands encompassed by the two cysteine residues. The oxidation of sulfhydril groups in Caf1M and DsbA,B,C by the active forms of oxygen produced by macrophages/monocytes (M) blocks the proper folding of Caf1M, consequently, stop the capsule formation at the site of contact with M. Its shedding facilit ates the interaction of molecular usher protein Caf1A (functioning as the high affinity hIL-1 receptor) with proIL-1α anchored to the M membrane. The acidification at the site of contact triggers off the expression of pH6 (PsaA) antigen functioning as IgG-binding receptor. It additionally attaches the pathogen to the M by the interaction of PsaA-IgG complexes with FcR. The tight contact of pathogen with M blocks the diffusion of Ca^{2}+ in periplasm and triggers off polarised secretion of Yops proteins using the type III secretion system. The Yops inhibit cytoskeletal functions and signal transduction pathways of M. The interaction of different strains of F. *tularensis* with human and animal IgG purified from non-immune sera was found. 63 kDa protein from outer membrane of F. *tularensis* ultrasonic strain 15, biovar *holarctica,* able to interact with human and rabbit IgG from non-immune sera, was isolated. Protein molecular mass was 63 kDa according to the data of SDS-PAGE in reduced conditions and 450 kDa, as estimated by gel-filtration in native conditions. Counted on CD spectrum data, the content of α-helices in protein molecule is 55%. DASM studies demonstrated that tertiary structure of protein was organized in two thermodynamic dimains melting independently of each other in cooperation way. During storage the protein was spontaneously degraded into two unequal domains with molecular masses of 37-38 and 25-26 kDa, correspondingly. Then the first domain was degraded to the domain with a molecular mass of 28 kDa that revealed an existence of the third domain with a molecular mass about 9-10 kDa. Recently, the gene of p63 was cloned and partly sequenced by V.M. Pavlov in the Institute of Applied Microbiology, Obolensk (personal communication). It was found that the sequence is identical to the independently cloned and sequenced GroEL homolog (molecular chaperone Hsp65) of F. *tularensis*. The gene was expressed in E. *coli* and the protein *was* purified to homogeneity. It was demonstrated by us that the monoclonal antibodies produced to the natural p63 effectively interacted with the recombinant protein. The amino acid composition of natural p63 expressed on the surface of F. *tularensis* and the amino acid composition of GroEL homolog of F. *tularensis* deduced from the nucleotide sequence of gene are practically coincided. These data as well as comparison of other structural characteristics of p63 and GroEL allow to conclude that p63 is GroEL homolog abnormally expressed on the surface of F. *tularensis*.

Conclusion: *Y. pestis* has a number of sensor mechanisms which involved molecular chaperones and sensed a response of M to the pathogen and adequatevely expresses the surface molecules from its anti-phagocytic arsenal. This arsenal helps *Y. pestis to* overcome an innate resistance of host and prevents an adequate adaptive immune response. The high level of homology of Hsp65 from different pathogenic bacteria can explain why the p63 protein *of F. tularensis* interacts with IgG from non-immune sera. This property of the bacterium to interact with crossreacted antibodies to Hsp65 can help it in attachment to M via Fc-receptors. The expression of GroEL homolog on the bacterial surface can explain the polyclonal expansion of γδ T cells observed soon after onset of tularemia in man. The ATPase activity of p63 can decrease the concentration of ATP intra M and as the consequence to decrease an oxidative-reduction potential of a target cell and to abolish an adequate phagocytic response to pathogen.

MOLECULAR TOOLS FOR THE STUDY OF TRANSCRIPTIONAL REGULATION *IN FRANCISELLA TULA*RENSIS

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Introduction: Induction of protein synthesis *in Francisella tularensis* in response to stress was first discovered by Ericsson et al. [1]. It is known that in a number of microorganisms stress protein synthesis is regulated by the substitution of DNA-dependent RNA-polymerase (RNAP) σ-subunit which play a leading role in the recognition of target promoters [2]. UP elements interacting with RNA-polymerase a -subunits and AT-rich sequence located immediately upstream to the -35 and -10 regions are also important for transcription initiation (3,4]. Taking into account AT-rich F. *tularensis* DNA content one can suggest that the latter factors will contribute essentially to *F. tularensis* DNA transcription initiation. At present no convenient molecular tools to study transcriptional regulation in *F. tularensis* are available. Therefore, this study was devoted to the construction of a vector for cloning of constitutive and inducable promoters of F. *tularensis* and characterisation of the promotors cloned.

Materials and Methods: *Escherichia coli* HB101, *F. tularensis* 15/10 (collection of SRCAM, Obolensk) *F. tularensis* 503 (collection of Gamaleya Institute of Epidemiology and Microbiology, Moscow) strains; pUC18, pML2.1, pFNL2OO plasmids [5-7] were used in this study. Cultivation of bacteria, preparation of plasmid and chromosomal DNA,

transformation of bacteria by plasmid DNA and construction of recombinant plasmids were carried out as described by Pavlov et al. [6]. Isolation of RNAP of *F.tularensis* 15/10 was performed according to Pomerantsev et al. [5]. Chloramphenicol treated white mice were used for selection of in *vivo* - derepressable *F. tularensis* promotores. F. *tularensis* DNA sequences for a promoter region analysis were obtained from GENE data bank.

Results: Studies on structure-functional properties of F. *tularensis* RNAP revealed high transcriptional peculiarity the enzyme towards the own DNA of the microbe. It was also found that F. *tularensis* RNAP subunit composition is similar to that of prokaryotic RNA-polymerases. In addition, molecular weight of a -subunit of F. *tularensis* was shown to be higher than that of a-subunit *of Escherichia coli.* Along with major a- subunit, F. *tularensis* RNAP also contains minor proteins, which probably function either as additional a-subunits or as transcription activators intrinsic for eucaryotic RNApolymerases.Analysis of primary sequences of regulatory regions of a number of structural genes of *F. tularensis* and genes exhibiting different expression in the microbe rarely revealed consensus -35 and -10 regions *of E.coli* and *Bacillus subtilis* promoters.

Vector construction was performed on plasmid *pFNL2OO* [6] by substituting cat-gene of Tn9 transposone possessing the own promoter for promoter-free cat-gene of Tn9 transposone with the retained Shine-Delgarno (SD) sequence [7]. Bsml, StyI,Xbal and BamHI sites were located immediately in front of SD sequence. All the sites might be used for cloning F. *tularensis* DNA promoter fragments. The constructed plasmid *pVP23 (6,4* Kb) was replicated and expressed tet-gene in F. *tularensis.* Sau3A *F. tularensis* DNA fragments incorporated in vector *pVP23* BamHI site immediately before promoter free cat-gene were studied. *In vitro* and in *vivo* promoter selection was performed according to the ability of clones obtained to survive in the presence of chloramphenicol. Studies on the relationship between environmental factors and activity of the cloned promoters are in progress now.

Conclusion:The plasmid vector *pVP23* for cloning of the constitutive and inducible promoter regions of F. *tularensis* DNA was constructed.

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FRANCISELLA TULARENSIS STRESS PROTEINS

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Bacterial cells respond to extreme changes in temperature and other unfavourable effects with reorganization of protein synthesis: synthesis of some polypeptides is decreased whereas stimulation of the synthesis of a set of specific proteins called stress proteins occurs. This phenomenon allows bacteria to survive under unfavourable conditions.

It is established that Francisella tularensis incubation under heat shock condition (42°C) induces synthesis of heat shock proteins similar to conserved chaperone protiens Gro EL and Dna K.

Investigation of F. tularensis capacity of exhibiting stress response in vivo was performed. Guinea pigs with subcutaneously implanted diffusion chambers (0,22 microns pores) were used as an experimental model. The chambers contained suspension of the F. tularensis 15/10 vaccíne or 543 virulent strains. A mixture of [^{14}C)-aminoacids was introduced into the chambers at various time intervals. After 24 hours the contents of the chambers were analyzed by electrophoresis followed by autoradiography for the presence of newly synthesized proteins. A sharp decrease in the number of viable cells of the F. tularensis 543 strain contained in the diffusion chamber was detected. Increased absorption of plasma proteins (predominantly albumins) by bacterial cells was also revealed. Analysis of the radiographs has shown that in vivo F. tularensis cells synthesize major polypeptides of approximately 65, 70 and 90 kDa. Polypeptides with similar molecular masses were revealed in: in vitro control experiments when the bacteria were subjected to heat shock.

A polymeric heat shock protein was isolated and purified from F. tularensís 15/10. The purification procedure included preparation of clarified lysate and chromatography on DEAE-cellulose and Ultragel AcA34. The material collected with gel filtration contained a homogenous 60 kDa protein. Electron microscopy revealed a characteristic star-like structures, similar to structures formed by Escherichia coli Gro EL.

The isolated polypeptide that can be induced under temperature stress conditions has been identified as an analoque of the Gro EL stress protein judged by molecular masses of the subunits, its polymeric structure and morphology seen in the elecrton microscope studies. Thus, F. tularensis is shown to be capable of exhibiting stress response in vivo and in vitro. A protein is isolated from F. tularensis cells which on the basis of the data obtained is identified as a stress protein belonging to the Gro EL chaperonine class.

LIPOPOLYSACCHARIDE STRUCTURE OF FRANCISELLA TULARENSIS MUTANT STRAINS

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Introduction: The great interest in the surface structures *of F.tularensis* is determined by their leading role in an expression of the virulence. The virulence decreasing has been shown to correlate with the alteration *of F. tularensis* lipopolysaccharide (LPS) structure (Khlebnikov et al., 1991; Markov et al., 1991; Sorokin et al., 1992; Pavlovich, 1993). The protective effect against tularemia of monoclonal antibodies (Mabs) specific for O-side chains of LPS implies that LPS may be an important immunogen (Khlebnikov et al., 1992,1993). The purpose of this study was the investigation of LPS structure of virulent and avirulent *F.tularensis* strains of different subspecies.

Materials and Methods: A few *F.tularensis* virulent strains (sub-species mediaasiatica, nearctica, holarctica) and their isogenic capsule-deficient mutants were used. LPS was prepared by the waterphenol extraction (Westphal, Jann, 1965) from the acetone-dried cells or the outer membrane treatment with proteinase K (Martinez,1989). LPS structure was analysed by means of SDS-PAGE with following silver staining and immunoblotting using the rabbit antiserum or Mabs specific for O-side chains of LPS.

Results: Comparative study of LPS preparations allowed to ascertain essential structural difference between LPS isolated from virulent strains and avirulent ones. LPS of parent virulent strains appeared as typical LPS of smooth Gram-negative bacteria (S-LPS), so it had the ladder-like structure whereas LPS of some cap- mutants appeared as core structure without O-side chains (R-LPS) and another ones had only one O-side chain (SR-LPS). Capsule-defective variants of F. tularensis denoted by us as cap ± produced S-LPS, however its O-side chains significantly differed from polysac-charide component of S-LPS of wild-type strain. This difference included the lowering of high mole-cular weight O-chain content accompanied by the appearance of additional low molecular weight polysaccharide molecules. Interesting results were obtained when analyzing the Pro K-digested cell lysates *of F. tularensis* cap- cells by immunoblotting with treatment of blotes with anti-F.tularensis LPS O-side chains Mabs kindly provided by V.S. Kchlebnikov. We found that two of four cap-strains contained intracellular O-units not exposed when analyzing LPS preparations isolated by usual methods. Besides that it was shown that strains of different subspecies had a variety of O-side chains distribution.

Conclusion: Above mentioned mutants are the convenient model to determine the significance of cellular surface structures in overcoming of non-specific host defence.

PHOSPHATASE ACTIVITY IN DIFFERENT SPECIES OF FRANCISELLA

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At present genus Francisella includes several species: F.tularensis, F.novicida, F.novicida-like and F.philomiragia. However, question about two last representatives remains disputable. That is why comparative analysis of biological, properties of different Francisella representatives may promote the solution of this problem. The purpose of this study was an investigation of phosphatase activity in bacteria of genus Francisella.

It was shown that all Francisella species had intracellular acid phosphatase which could be revealed only after disruption of cell wall in distilled water. However, the level of phosphatase activity was different in various species of Francisella: F.tularensis subspecies mediaasiatica showed the largest activity, nearctica subspecies of F.tularensis and other representatives of Francisella - medial one, and holarctica strains of F. tularensis had either minimal or zero activity. The lysis of bacteria with sodium dodecylsulfate revealed the increase of phosphatase activity in nearctica and mediaasiatica strains of F.tularensis but this detergent completely inhibited the enzyme of holarctica strains and other Francisella representatives. Higher phosphatase activity in all strains including holarctica cultures with zero activity could be registered after treatment of microbes with tweens. Our studies allowed to suppose that while all Francisella strains had two forms of enzyme: membrane-bound and periplasmic ones, subspecies holarctica of F.tularensis had only membrane-bound form. The increase of phosphatase activity in the case of the tween treatment was determined by intracellular penetration of substrates and extracellular exit of periplasmic form of the enzyme. That is why the enzyme activity of strains which had two forms of phosphatase appeared in supernatants and cellular fractions while holarctica strains revealed only cell-bound activity. Multilocus enzyme analysis showed that Francisella phosphatases were characterized by different electrophoretic mobility.

Thus, the present investigation revealed that representatives of Francisella genus produce phosphatases which differ by the localization in bacterial cell, sensitivity to sodium dodecylsulfate and electrophoretic mobility.

COMPARATIVE STUDIES OF ULTRASTRUCTURE AND MORPHO-POPULATION PROPERTIES OF THE FRANCISELLA SPECIES.

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Morphology and ultrastructure of cells and structure of microbial populations of different Francisella species have been thoroughly studied /1,2/. Less is known about the ultrastructure of cells and morpho-population properties of F.novocida-like and F.philomiraga. Comparative electron-microscopic studies of different Francisella species is the objective of the present work.

It has been found out that F. novocida-like, F. philomiraga F9693 and F. tularensis strains 503, 15.3M and Acole form heteromorphous populations. In F.novocida-like populations grown on liquid and solid media cells of different forms are revealed: cocci, coccobacilli, short and long bacilli as well as cells of abnormal form of a size of 0.7 - 0.6/0.9 - 0,6/1.2- 1.4 μm.

Almost 70% of cells of F.philomiraga are cocci and oval cells of a size of 0.7 and 0.6/0.9 μm; the rest are short and long bacilli of a size of 0.5/1.2 and 0.5/1.8 μm.

In all growing old populations of Francisella the formation of small cells (0.1-0.3 μm) and cells with processes was observed. The cells of all species were found to be covered with capsule-like formation 0.1-0.3 μm thick. In most cases this formation promotes the aggregation of microbes into groups of 3-30 individuals each.

The cells of all Francisella species have smooth outer membrane, a weakly developed peptide glycanic layer and comparatively smooth cytoplasmic membrane. The nucleoid occupies 50-60% of the cell volume and has a fine structure in all the species. The cytoplasm has a loose structure and contains ribosomes, polysomes, fibrilar and globular biopolymeres and different membrane formations in its matrix. The cells of all Francisella species divide through budding, cytoplasm invagination or through the formation of the septum. In F.novocida-like the formation of daughter cells was observed in the cytoplasm of parent ones due to the segmentation of a part of the nucleoid and mother cell cytoplasm through the formation of new cytoplasmic membrane.

General morpho-population and ultrustructural properties of F. novocida-like, F. philomiraga and F.tularensis, in particular heteromorphosis of the populations, small size of the cells as compared to other microorganisms, availability of capsule-like formation aggregating the microbial cells, smooth outer membrane, loose structure of the cytoplasm, nucleoid of large size, weakly-developed intracytoplasmic apparatus, specific ways of cell division enable one to consider that all the species studied belong to Francisella genus.

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MONOCLONAL ANTIBODY ANALYSIS OF LIPOPOLYSACCHARIDE FROM *FRANCISELLA TULARENSIS*

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Introduction: The ability of monoclonal antibodies to be utilized in the analysis of antigenic structure is just now beginning to be appreciated in microbiology. These studies have demonstrated the application of monoclonal antibodies (mABs) for the definition of the antigenic structure of lipopolysaccharide (LPS) from *Francisella tularensis.*

Materials and Methods: To study monoclonal antibody binding to LPS ELISA and SDS-PAGE and Western immunoblotting analysis were used.

Results: In this study we selected a hybridoma which is producing FB11x mABs (IgG_{2a}, subclass) recognizing a determinant of LPS moieties. In preliminary experiments FB11x have shown potent protective activity against *Francisella tularensis* strain 15. LPS are amphipathic molecules composed of a lipid part, termed lipid A, and heteropolysaccharide subdivided into the O-specific polysaccharide chain and the core oligosaccharide. ELISA inhibition systems and immunoblotting analysis identified the major antigenic determinant for FBIIx mABs contained in the main carbohydrate chain of macromolecule. These antibodies failed to reoognize the site on rough-type LPS which had no O polysaccharides. It is known that the antigen site recognized by antibody is likely to be determined by one monosaccharide residue or oligosaccharide moiety of the replicated unit of O polysaccharide. It was shown that O polysaccharide *Francisella tularensis* have following structure:

-4)-α-D-GalNAcAN-(1-4)-α-D-GalNAcAN-(1-3)-β-D-QuiNAc-(1-2)-β-D-QuiFm-(1-.

To determine which residue(s) in the O polysaccharide was an epitope for FB11X we tested specific binding of different sugars and oligosaccharide fragments obtained from the O polysaccharide using acid hydrolysis by competitive ELISA. Studies with a wide variety of carbohydrates including D-glucose, D-galactose, L-rhamnose, 2-keto-3-deoxyoctanoate and D-glucoheptose failed to inhi-

bit this ELISA. D-Galacturonic acid and N-acetyl-D-galactosamine, which are similar in design to aminouronic acids (Ga1NcAN) presenting in LPS, partially inhibited the mABs in concentrations as high as 100 mg/ml. Studies of different oligosaccharide fragments indicated that complete inhibition was achievable using oligosaccharides containing 2-acetamido-2-deoxy-D-galacturonamide.

Conclusion. These data indicate that N-acetyl-D-galacturonamide residue might be the epitope recognized by FB11x mABs. Future studies are planned with these preventive mABs in an attempt to define the biological function of LPS *Francisella tularensis.*

LOCALIZATION *OF F.TULARENSIS* IGG-BINDING COMPONENTS AND CORRELATION BETWEEN THEIR CONTENT AND VIRULENCE.

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Introduction: In this work the binding of bacteria *F.tularensis to* immunoglobulins, isolated from human and rabbit non-immune sera was studied A 63 kDa protein, a possible homologue of GroEL (molecular chaperone Hsp 65), is one of the main bacterial components *of F.tularensis* responsible for interaction with IgG. The interaction of bacteria *F.tularensis* with "normal" immunoglobulins can be explained by the presence of the antibodies, cross -reacting to conservative stress proteins of different bacteria, in the preparations of the latter.

Materials and methods: The 63 kDa protein (p63) on the surface of alive *F.tularensis* cells of vaccinal strain 15 (type B) and virulent strains 503 (type B) and Schu (type A) was detected by immunoelectron microscopy using intact human IgG, labelled with horse-radish peroxidase. The amount of p63 in different strains was estimated using the immuno-enzymatic method. The number of the bound IgG molecules, labelled with peroxidase, was calculated using a Scatchard plot and taking into account the natural peroxidase activity in the strains and the difference in the size and area of the cells in the vaccinal and virulent strains. The culture of St. *aureus Cowan I* cells carrying protein A on their surface, interacting with normal immunoglobulins, was used as a control.

Results: It has been shown that bacteria *F. tularensis* interact with immunoglobulins, isolated from human and rabbit non-immune sera. In 10% of the bacteria of the vaccinal strain and in 60% of the virulent strains a distinct thick contour, formed due to the binding of IgG labelled with peroxidase to p63, was observed on the cell perimeter. The calculation of the number of p63 molecules per 1 $m\mu^2$ of the bacterium surface showed that there were 20 000 protein molecules in the mentioned area of the vaccinal strain and 60 000 molecules were present on the area of the virulent strains.

Conclusion: Thicker localization of p63 on the surface of the virulent bacteria F. *tularensis* provides the latter effective binding to IgG. As a result the cells *of F. tularensis* virulent strains acquire the possibility to be actively bound to phagocytes. The expression of GroEL homologue on the surface of F. *tularensis* can explain polyclonal expansion of $\gamma\delta$T-cells, which is observed soon after a man catches tularemia. The effect of p63 on phagocytic response of the cells, infected with *F. tularensis,* is not excluded.

CORELLATION BETWEEN FRANCISELLA VIRULENCE AND RESISTANCE TO TEMPERATURE AND BACTERICIDAL ACTION OF NORMAL SERUM

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Up today rapid determination of Francisella virulence in vitro remains not enough studied. It is known that the bactericidal action of normal blood serum is one of the F.tularensis virulence criteria (Pavlovich e.a., I996). There are also reports that F.tularensis mutants possessing temperature-sensitive phenotype are proved to be avirulent and some of them immunogenic (Bhatnagar e.a., 1994; Getachew e.a.,I995; Kormilitsyna, Meshcheryakova,1996). As to other Francisella species similar data are absent.

We used two in vitro tests for comparative research of Francisella strains differed in virulence: 1) resistance determination to the bactericidal action of normal rabbit serum (NRS); 2) resistance to 42°C cultivation temperature. Among Francisella strains were studied: F.tularensis (69 virulent, 3 vaccine, 7 avirulent); F.novicida (-like) (3); F. philomiragia (2).

The following results were obtained. All F. tularensis natural virulent strains (LD_{50} for mice and guinea pigs is 1 m.c.) showed resistance both to the bactericidal action of NRS and to 42°C cultivation temperature (tr 42). Vaccine strains (or variants) as well as individual clones differed in these two tests according to the level of their <<residual>> virulence. Thus the vaccine strain 15/10 possessing <<residual>> virulence (LD_{50} -10^2-10^3m.c.) was resistant both to NRS and temperature 42° C (tr 42) while attenuated variants which possessed reduced virulence in mice (LD_{50} -10^5-10^7m.c.) remained resistant to NRS but lost their ability to grow at 42°C temperature and some of

them - even at 41°C (ts 41). Completely avirulent and nonimmunogenic strains showed high susceptibility to NRS and temperature 42°C (ts 42).

Similar tendency was revealed in two other Francisella species strains differed in virulence. F.novicida Utah 112 - virulent in mice (LD_{50} -10^4 m.c.) and Syrian golden hamsters (LD_{50} -1 m.c.) was found to be resistant both to NRS and tr 42°C, whereas completely avirulent F.philomiragia strain F 9017 was sensible in both tests.

Thus the determination of resistance to the bactericidal action of normal serum and cultivation temperature can serve tentative in vitro criteria of Francisella strains virulence.

DEPENDENCE OF THE MAGNITUDE OF THE EFFECTIVE DOSE ON DISPERSIBLE COMPOSITION OF AEROSOLS OF A LIVE TULAREMIA VACCINE

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Earlier we have experimentally substantiated higher effectiveness of aerosol immunization with a live tularemia vaccine as compared to subcutaneous administration.

At the present stage of the work, we have investigated immunologic peculiarities and the efficacy of immunization of rabbits with a finely dispersed (mass-median aerodynamic diameter of d_{mma}=2,3 μm, standard deviation δ_g=1,6) and a coursely dispersed (d_{mma}=6,0 μm, δ_g=1,8) aerosols of a live tularemia vaccine (LTV), 15 NIIEG strain.

It has been established that the finely dispersed aerosol of LTV at a sedimentation profile in the nasopharyngeal (NP), tracheobronchial (TB) and alveolar (A) regions of the respiratory tract (NP:TB:A = 77:13:10) displays a more pronounced activating action on blood phagocytes, especially at the level of the esterase activity. At the same time, the coarsely dispersed aerosol (with a sedimentation profile of NP:TB:A=94:3:3) makes greater impact on the activation of the mechanisms involved in the secretion of myeloperoxidase and nonspecific esterases into the blood. Taking into account that enhanced secretion of enzymes favours a drastic drop of their intracellular concentration, it can be suggested that the administration of the finely dispersed aerosol of LTV appears to be more favourable for complete functioning of the initial (nonspecific) link of the immune system. Immunization of rabbits with a finely dispersed aerosol at doses of $5x10^4$ - $1x10^6$ live microorganisms/cell contributed to a more intensive accumulation of specific antibodies, during which for prolonged periods following vaccination, similar serological shifts (1:320, 1:640) were observed for the coarsely dispersed aerosol at 13-14 times higher doses. Immunization efficacy was evaluated under the conditions of challenging the rabbits with aerosolized virulent Schu-S4 strain at a dose of $(4,5\pm1,3)x10^3$ LD_{50} (1 LD_{50} = 126±13 live microorganisms/cell at d_{mma} = 5,5 μm, δ_g = 1,6). It has been found that ED_{50} for the finely dispersed aerosol that amounted to $(2,3\pm0,9) \times 10^5$ live microorganisms/cell was 24 times lower than that for the coarsely dispersed aerosol. Thus, it has been shown that in the event of using finely dispersed vaccinal aerosols it is possible to lower the effective immunizing doses of LTV considerably.

EXPERIMENTAL MODELS OF AEROGENIC TULAREMIA

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It is known that when valuating the efficacy of the means of prophylaxis of infectious diseases under the conditions of aerosol challenge, consideration must be given to the inhaled pathogenic microorganisms dispersible composition that determines the sites of preferential application of particles in the respiratory tract and ultimately the magnitude of the effective infectious dose.

We have developed experimental models of aerogenic tularemia which are distinguished by the density (profile) of microbial particles sedimentation in the nasopharyngeal (NP), tracheobronchial (TB) and alveolar (A) regions of the respiratory tract. Tularemia infection was reproduced in outbred white mice weighing 18-22 g and Shinshilla rabbits weighing 2.7-3.6 kg under conditions of a horizontal dynamic facility. A suspension of a 48-hour culture of the Schu-S4 strain grown on the yolk medium at 37°C was used. Animals death record was kept for three weeks, and the LD_{50} value was determined by the probit analysis method. Inhaled doses of the pathogen were evaluated with regard to the coefficients of general and local retention of particles in the respiratory organs of the animals. It has been established that the LD_{50} value for intact white mice challenged with an aerosol with a mass-median diameter of d_{mma} = 2,6 μm and standard deviation of δ_g=1,6 (with a sedimentation profile of NP:TB:A = 70:13:17) was 20±3 live microorganisms/cell, whereas after the administration of aerosol of d_{mma} = 5,5μm, δ_g=1,6 (with a sedimentation profile of NP:TB:A = 83:9:8) it was 35±4 live microorganisms/cell, i.e., a change of 1.5 times. When rabbits were challenged, it was found

that the LD_{50} value for aerosol with d_{mma} = 2,6 μm, δ_g=1,6 and sedimentation profile of NP:TB:A = 80:11:9 was 79±11 live microorganisms/cell, whereas for d_{mma} = 5,5 μm, δ_g=1,6 and sedimentation profile of NP:TB:A = 90:6:4 it was 126±13 live microorganisms/cell. The results of the investigation proved once again the necessity of considering the dispersible composition of aerosols during experimental reproduction of aerogenic infections.

MODEL INVESTIGATIONS ON SOME PROTECTIVE MECHANISMS IN TULAREMIA INFECTION AFTER AEROSOL IMMUNISATION WITH LVS

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Introduction: The investigation was targeted to analyse the activation of different protective mechanisms to tularemia infection in immunised with LVS spites and correlation with biology characteristic of the model animal.

Materials and Methods: 1)Strains - F.tularensis-LVS and F.tularensisSrebarna19. 2) We used microbe isolation and titration techniques, preparation and cultivation methods for phagocyting cells, immunofluorescent method. 3) guinea pigs, rats and rabbits were used.

Results: We assessed comparatively the bacterial clearans from the blood of tularaemia microbe in both intact and immunised animals. In ten time periods during the first hour and after 24h following intracardial bacterial inoculation, we found significant difference in the dynamics of persistens and clearans.

We followed up the dynamics of intra- and extracellular bacteria in peritoneal lavag after i.p. inoculation of strain F.tularensis-Srebarna19 in the model of intact and aerosol immunised "Wistar" rat and guinea pigs. In immunised animals there was significant predomination in intracellularly situated bacteria. In intact animals, which meet the antigen for the first time, most of the bacteria were cyclical in the extracellular fluid. This is probably the result of the obtained destruction of the phagocytes.

A difference was found in infectious sensibility in dependence on invasion roots.

Conclusion: The results suggest that in the course of tularemia not only system immune reactions play important role but so do local protective me-chanisms. The grade of this reaction depends to a great extent from infectious sensibility of host animal.

INTRACELLULAR FAITH OF F.TULARENSIS-LVS IN ALVEOLAR MACROPHAGES IN VIVO AFTER AEROSOL IMMUNISATION

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Introduction: Intracellular faith of F.tularensis - LVS in alveolar macrophages was investigated in experimental conditions on different terms after aerosol immunisation aiming to follow up the persistence of the microbe, and its destruction and clearans.

Materials and methods: Investigations were conducted in the guinea pig model with exposition in chamber of dynamic type with controlled parame-ters: t-l8°C, Hg-80%, Q/V-1:4.

Bacterial strain: Francisella tularensis-LVS obtained from Institute Gamaleja, Tularemia Laboratory- Moscow.

The effective dose was D_{eff}=6.10^5m.c.

Microbial quantity was controlled bacteriologically in lungs, tracheobronchial lymph nodes, spleen and liver and using immunofluorescence on stamp and slide preparations, and in bronchoalveolar lavage.

Results: F.tularensis-LVS proliferates and persists mainly in the lungs (7- 10d) and regional lymph nodes for about 2 weeks, in the spleen - between 3 and 5 days.

On immunofluorescent lung preparations we founded in early terms (0-5d) intact morphology of microbes intracellulary situated. In the course of the process we observed single or group bacteria phagocyted by alveolar macrophages. Ju an early stage 3-5d the morphology of the agent was intact but la-ter begins a destruction period. About d10-15 we could not find in practice intact bacteria. The picture is similar to "star sky" constructed from antigen diffuse fragments. In the next stage antigen disappears.

Conclusion: The information obtained for the character of the interaction of micro-macro-organism on cellular level is discussed as a part of immune response induction and protection grade.

DETECTION OF *FRANCISELLA TULARENSIS* IN ULCERS OF TULAREMIA PATIENTS USING PCR

Introduction: The diagnosis of human cases of tularemia is usually confirmed by the demonstration of an antibody response to *Francisella tularensis*, occurring about two weeks after onset of disease. Due to a high risk of laboratory infection, cultivation of the causative agent tends to be avoided. During an outbreak in Sweden, the polymerase chain reaction (PCR) was evaluated for diagnosing the ulceroglandular form of tularemia.

Materials and Methods: In August and September 1995, tularemia occurred in two areas of central Sweden. Through general practitioners, wound and blood samples were obtained from patients seeking advice with suspected ulceroglandular tularemia. Fifty patients (2-81 years old, mean, 44 years, 28 females, 22 males) were included, none of whom had received antibiotic treatment before the day of visit. The method for sample preparation was based on the binding of DNA to uniform glass beads (Glass milk, BIO 101, La Jolla, Calif.) in the presence of the chaotropic nuclease-inhibitor guanidine-isothiocyanate (Sigma, St. Louis, MO). Samples, 5 μl, were subjected to PCR using primers specific to sequences of the 16S rRNA gene and a gene encoding a 17-kDa lipoprotein of *F. tularensis* LVS. Separately, samplex were also subjected to amplification with primers specific to introns of the human β-actin gene. To confirm the identity of the 17-kDa lipoprotein gene product, Southern blotting was performed.

Results: Out of 50 patients seeking attention during the tularemia outbreak, serology was confirmative in 40 and negative in 10. By culture, 10 of 50 wound samples showed growth of F. tularensis. These 10 samples all originated from patients who were serologically confirmed.

PCR was performed on wound samples from all 50 patients. Of those 40 subjects who were serologically confirmed, 29 (73 %) showed successful amplification of the 17-kDa lipoprotein gene of F. tularensis and 20 (50 %) of the 16S rRNA gene. All the latter 20 patients belonged to those from whom the 17-kDa lipoprotein gene was identified. By Southern blot hybridization, the identity of the amplified 17-kDa lipoprotein gene was consistently confirmed. Among those 10 subjects who were negative by serology, one showed detectable *F. tularensis* DNA. In this patient, both *F. tularensis* genes were detected and the patient showed a clinical picture typical of tularemia. Transportation time did not seem to decisively affect the outcome of the analysis.

Conclusion: In 29 of 40 (73 %) serologically confirmed tularemia patients, *F. tularensis* DNA was successfully amplified. Considering the limitations of current diagnostic procedures, PCR may become useful for the early diagnosis of tularemia.

CHARACTERIZATION AND CLASSIFICATION OF DIFFERENT STRAINS OF FRANCISELLA TULARENSIS ISOLATED IN CENTRAL EUROPE

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Introduction: *Francisella tularensis (F.t)* is widely distributed in the Northern hemisphere. *F.t.subsp. tularensis* occurs in North *America, F.t.subsp. palaearctica(holarctica)* occurs in Europe,Asia, North America and F. t. *subsp. mediaasiatica was* found only in central Asia of the former Soviet Union.In 1963-1992, during surveillance activities in Slovakia, 205 F.t. strains were isolated and classified by basic markers of subspecific taxonomy. The majority of isolates were characterized as *F.t.subsp.palaearctica (holarctica, biovar* II.Ery^r), while some strains,isolated from ectoparasites in the Danube region near Bratislava in 1986-1988, had biological properties of F.t. *subsp. tularensis.* First findigs of the highly virulent strains of F.t.in Europe indicate a serious event from the epidemiologic point of view. The new data promoted us to a wider comparative study of F.t.strains isolated mostly in 1990- 1996 from different regions of Central Europe.

Materials and methods: We examined 53 F.t. strains isolated in our laboratory from Slovakia, Austria, Czech Republic and Hungary. These strains were compared with 5 reference strains and 4 type strains from Slovakia and characterized by biochemical and virulence tests, as well as by sensitivity to erythromycin.

Results and conclusions: The comparative study revealed that 52 isolates had biologic properties *of F.t.subsp. palaearctica (holarctica, biovar* II). One strain isolated from *Ixodes ricinus* ticks in Austria (1990) had properties *of F.t.subsp. tularensis. The* strain fermented glycerol, citrulline, was sensitive to erythromycin and was highly pathogenic for rabbits, similarily as two type strains *of F.t. subsp. tularensis* isolated in Slovakia. This further isolation of F. *t. subsp. Tularensis* in Europe confirmed our first findings of these strains in Slovakia and indicate the requirement of systematic surveillance in Central Europe.

STUDY OF A MIXED NATURAL FOCUS OF TULAREMIA AND FIELD FEVER (1977-1994) IN SLOVAKIA AND INFLUENCE OF RODENTICIDE APPLICATION

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Introduction: In a known endemic area of tularemia and field fever in West Slovakia, a mixed natural focus of both infections was submitted to a systematic surveillance during the years 1977-1994. With regard to an epizootic of tularemia in this focus in 1977-1978, a repeated application of a rodenticide was realized within the years 1978-1980.

The study was based upon investigation of changes in the number and distribution of different species of small terrestrial mammals (s.t.m) after

deratization and on observation of the impact of these changes on the persistence of the natural focus.

Materials and methods: The natural focus is an abandoned orchard situated between fields. The application of the rodenticide (5% granulated zinc phosphide) started during epizootic activity of tularemia in autumn 1978 and was repeated together 5 times in 6-months intervals. Animals-s.t.m. were live-trapped in intervals of one or three months and examined for the presence of *F.tularensis* and *leptospires by* usual culture methods. The isolated strains were identified by standard methods.

Results and conclusions: The chemical intervention reduced the number of s.t.m.and changed the structure of species significantly: the primarily dominant M.arvalis was suppressed and replaced mostly by C.glareolus.These changes observed during the whole period of investigation led to the suppresion of tularemia for a long time. On the other hand, occurrence of field fever was not apparently influenced even though a distinct decline of this infection among s.t.m. was observed. Fourteen years after deratization, a significant increase of s.t.m.occured and was followed by reactivation of both infections in the natural focus. Importance of different species of reservoir animals in the maintenance of both infections in the focus is discussed.

NONCULTURABLE FORMS OF FRANCISELLA TULARENSIS AND THEIR POSSIBLE EPIDEMIOLOGICAL SIGNIFICANCE

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Introduction: Many bacterial infectious agents are known to be able to be preserved in the natural environment as peculiar forms that do not grow on routine culture media but can recover their proliferative activity under certain conditions (Colwell, 1993). It is not known if F. tularensis can exist as such unculturable but viable forms.

Material and Methods: The possibility of F. tularensis transition to an unculturable state was studied using the Schu strain (biovar nearctica). Bacterial cells (10^7 cells/ml) were placed in flacks containing either deionized water or deionized water plus 0.15 M NaCl. The viability of the cells was determined by daily plating the samples on T-agar followed by investigation in PCR using genus-specific primers (Romanova et al., 1994, 1995).

Results: It is found that growth of the cells in the deionized water (28° and 37° C) stopped on the 1 st day after plating, whereas the cultures from the cells incubated with the addition of NaCl at 37° C were recovered up to day 3 and those grown at 28° C up to day 7. Though the growth was not detected after further plating, PCR results remained positive for 22 days (period of observation). Samples from the flacks containing water plus 0.15 M NaCl removed on the 8-th day and inoculated into white mice at a dose of 0.5 ml i.p. were avirulent for the animals. PCR analysis of the total DNA obtained from the internal organs of the mice killed on 8-10 days gave positive results. During routine investigation of tularemia natural foci in the Rostov district area in winter 1997 about 300 samples (from water, rodents, straw and ticks) were tested for tularemia. 47 of them including samples from mite ticks were positive in PCR. F. tularensis strains were recovered in 8 cases after repeated passages through mice.

Conclusion: Starvation-induced stress may promote F. tularensis transition to the unculturable state, which may enable the bacteria to persist in the natural environment. Cells of F. tularensis Schu lost virulence readily after the transition to the unculturable state and this may indicate that the process is under genetic control activity through switch-off (stress) - switch-on (passages through susceptibile animals, cannibalism) mechanism. Divergent results of bacteriological analysis and PCR obtained during the epizootological surveillance may be explained by the F. tularensis capacity to exist in the natural environment as unculturable (avirulent) forms that are difficult to detect by traditional procedures. Isolation of typical cultures from the PCR + field material after 3-4 passages through mice provides evidence that the pathogenic S-form is not the only natural form of F. tularensis. PCR-based preliminary selection of samples obtained from field material followed by bacteriological analysis allows to increase the efficiency of epidemiological surveillance of tularemia in natural foci.

UPDATE PROBLEMS OF TULAREMIA PROPHYLAXIS IN RUSSIA

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Tularemia is widely distributed in Russia. It occurs in the NorthWest,Centre, South and South-East of the European part and also in Siberia, Far East of the Asian part. As for the other areas the focal territories are smaller, confined mainly to river valleys, in foothills and some mountain districts.Tularemia natural foci differ on the strength of their epidemiological, epizootological and ecological peculiarities. They manifest considerable stability and can exist indefinitely long if not affected by radical man-induced changes.

During the last decade (1987-1996) tularemia has been reported mainly as sporadic cases or epidemic outbreaks of moderated intensity. The number of cases varied from one year to another but did not exceed 400 cases a year, as a rule only in nonvaccinated people. This is many times less than the morbidity rate before 1950, i.e. prior to the application of vaccination.

Recently most of patients were infected either as a result of contacting animals, or bites of mosquitoes, or through water and other contaminated substrates. The ulcero-glandular, glandular and pharyngeal clinical manifestations were mainly reported. Over 70% of the patients were engaged in urban areas while almost 90% of cases were engaged in agricultural areas in earlier survey period.

Regular vaccination of the population with the effective live vaccine by Gaisky conducted together with other measures ensured radical reduction in the morbidity rate of this disease. The tularemia live vaccine is currently among the best vaccines as regards efficiency and longevity of protective immunity. It was calculated that the vaccination of people prevented against infection no less than 10 000 cases a year. Vaccination is applied among residents of areas with natural foci where people may be exposed to contact with the pathogen. Specific attention is given to occupational groups: hunters, trappers, farm workers and so on. In the eighties-nineties the number of vaccinated (and repeatedly vaccinated in 5-7 years) annually achieved 1,5 million.

The immunity in vaccinated persons is regularly controlled by means of modern immunological methods, while new prophylactic and diagnostic preparations are developed.

In addition to the vaccination the following prevention measures are used: improvement of the focal natural territories; protection of water sources, food products etc.; health education of people in focal areas.

BACTERICIDAL AND DISINFECTIONAL PROPERTIES OF NEW PREPARATIONS IN REGARD TO FRANCISELLA TULARENSIS.

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Currently peroxide- and chlorine-containing preparations are widely used for disinfection in medical institutions and microbiological laboratories as well as in the places of infectious disease bursts. Along with high bactericidal and sporocidal activities and stability on storage, the preparations have a number of disadvantages: they cause metal corrosion, chemical burns of skin, dermatitis, etc. In the last few years highly effective and safe disinfectants composed of active substances in combination with useful additives have been developed.

Bactericidal and disinfectious properties of the preparations and mechanism of action of new disinfectants, such as metacid, grylen, a composition on the base of catamine AB, stabilized sodium hypochlorite, a composition on the base of calcium hypochlorite, desoxon-4, cyaref on Francisella tularensis have been studied by different microbiological, biochemical and biophysical methods.

It was found that all the preparations studied, except for metacid, exhibited high bactericidal activity. Metacid, which has a polymere structure, is not capable of inactivating F.tularensis, which is far from being resistant to chemical and physical factors. Antimicrobial activity of the preparations was dependent to a variable degree on pH, temperature and protein admixtures of the medium. The curves of death rate of the microbes in the suspension containing subbactericidal concentrations of the preparations are presented in the report. The most part of the microbes dies in the first seconds of the contact with disinfectants. The data on the bacteridical activity of the preparations on the surfaces artifcially contaminated with the microbes may well be the basis for the development of various disinfection regimes.

Physical and chemical properties of the preparations, namely pH, viscosity, stability, corrosion activity with regard to construction materials have been studied.

It was found that peroxide-containing preparations provide membranotropic effect and cause lethal structure-functional disorders in the cytoplasmic membrane of the microbes. Chlorine-containing preparations operate at the level of enzymatic apparatus of the microbial cells, inducing structure damage in the nucleoid, cytoplasm and membrane apparatus of the cell.

Thus, the mechanism of action of new disinfectants and physico-chemical properties of them were studied. Conditions and parameters of the disinfection regimes were developed. It was concluded that the preparations studied can be recommended for use in medical institutions, microbiological laboratories as well as for disinfection in infectious disease foci.

THE INCIDENCE OF TULAREMIA IN COVERAGE OF INFECTIOUS DEPARTMENT OF NITRA HOSPITAL.

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Introduction: Tularemia is characterized as anthropozoonosis with natural focal centre. Its occur-

rence has been observed in Nitra and its surroundings.

Materials and Methods: We treated 90 patients with tularemia between January, 1990, and December, 1996. In 1995 an increased incidence of this disease had character of epidemics and developed due to the amplification of small rodents, higher morbidity and mortality rate non-domesticated hares.

Results: The age of our patients was from 18 months to 74 years. 47 patients (52,2%) had ulceroglandular form of tularemia, 17 patients (18,9 %) had pulmonary form, 14 persons (15,8%) had oroglandular form, 9 persons (IO,O%) had typhoidal form, 2 patients (2,2%) had occuloglandular form and 1 patient (l,l%) had gastrointestinal form. The contact with domesticated or non-domesticated hares was occurred in 41 persons.

Conclusion: We want stress the importance of the detailed epidemiological history in febrile patients, in patients with unilateral tonsillitis, with nodular syndrome, with atypical pneumonia who don't improve during the treatment with beta-lactamases antibiotics.

BASIC PARAMETERS FOR AEROGENIC MODEL OF INFECTION AFTER AEROSOL APPLICATION *OF FRANCISELLA TULARENSIS* LVS IN MICE

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Introduction: Aerogenic and transdermal infections are the natural ways for spreading of tularemia. Transdermal application of microbes has been extensively used for application *of Francisella tularensis* live vaccines in animal models and also in humans. There are only limited knowledge about the pathophysiological and immunological consequences of aerogenic infection with both wild *Francisella tularensis* microbes and *Francisella tularensis* live vaccines. The aim of the study presented herein was to answer the question if the aerosol exposure of macroorganisms is the suitable model of natural aerogenic infection.

Material and Methods: The chamber "nose only" with flow rate 6 l/ min. for aerosol application *of Francisella tularensis* LVS microbes to mice was used throughout the study. The particle size of aerosol, the inhalation to deposition ratio, LD_{50}, and minimal infective dose needed for generalisation of infection were used as parameters for aerogenic model of infection.

Results: Using the pneumatic syringe atomiser the aerosol with mass median diameter (MMD) of 2.63±0.812 qm with geometric standard deviation 1.833±0.155 was obtained. The deposition *of Francisella tularensis* LVS microbes in murine lungs was 25% under the parameters of aerosol. The LD_{50} parameter for C57B1/10 and Balb/c inbreed strains of mice differing in innate resistance to F. *tularensis* LVS was calculated to be 9.00×10^2 and 2.55×10^1 respectively. Moreover, only one live microbe after deposition in the lungs of exposed mice is sufficient for generalisation of infection. The mice that survived the primary aerosol infection expressed the defence mechanisms that are able to limit the proliferation of microbes delivered by secondary aerosol challenge in the lungs.

Conclusion: The defined application of microbes to lungs by aerosol is possible using our "nose only" system. In spite of generalisation of F. *tularensis* infection the infected mice survived low doses of primarily applicated infective aerosol and developed protective defence mechanisms. The described model of aerogenic infection will allow to study in detail local and general immune mechanisms participating in resistance towards air-borne infections.

THE POSSIBLE ROLE OF THE REACTIVE OXYGEN AND NITROGEN INTERMEDIATES IN EARLY STAGES OF F. *TULARENSIS* INFECTION AND INFLUENCE OF BCG LOCUS ON RESISTANCE TO INFECTION.

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Introduction: Natural resistance to infection by intracellular bacterial pathogens, most pronounced early during infection, reflects the capabilities of the molecules and cells of the innate immune system of the host including its genetics to control the rate of intracellular growth of bacteria. On the other hand, infectious invaders have evolved strategies to incapacitate or evade the innate and/or adaptive immune system. This study was designed to analyse the role of reactive oxygen and/or nitrogen intermediates (ROI and /or RNI) together with the expression of macrophage antioxidant proteins and their possible relationship to early-phase resistance to *Francisella tularensis* LVS infection in mice.

Material and Methods: The *Bcg* (B10.A.Bcg^r and C57B1/10) *or Lps* (C3H/HeN and C3H/HeJ) congenic pairs of mice were infected by sublethal dose (10^2 CFU, s.c.) of *Francisella tularensis* LVS. The production of ROI and RNI by peritoneal ad-

herent cells, and the number of CFU per spleen were measured in the course of 10 days after the challenge of mice. Differential protein expression in macrophages was analyzed using two-dimensional gel electrophoresis.

Results: The degree of resistance to F. *tularensis* LVS infection revealed by determination of CFU in spleen of infected mice decreased in order C57B1/10, B10.A.Bcg^r, C3H/HeN, and C3H/ HeJ mice with the most pronounced difference (2 log) between C57B1/10 and B10.A.Bcg^r mice. Only C57B1/10 mice were capable of parallel but intermediate production of ROI and RNI on day 5 after infection. On the other hand, BIO.A.Bcg^{r}and C3H/HeN mice were very close each other in regard to their degree of resistance, increased expression of macrophage proteins Bcl-2 and Mn-SOD, and decreased production of ROI or RNI on day 5 after the infection that was followed by remarkable enhancement between day 7 and 10 in comparison to C57B1/10 mice. Nevertheless, the time course of the onset of production of ROI and RNI was quite opposite between these two mouse strains. The C3H/HeJ mice were unable to produce ROI and the defect in regulation of the level of Bcl-2 protein might contribute to the susceptibility of this strain of mice to F *tularensis* LVS infection. Conclusion: Overall, these parameters may suggest importance of the coordination between ROI and RNI in early-phase resistance to F *tularensis* infection and demonstrate that *Bcg* locus influences resistance to F. *tularensis.* The question of adaptation of intracellular pathogen to the stressful enviroment inside the host and the capability to subvert host processes remains open.

CYTOKINE GENE EXPRESSION IN NAIVE AND IMMUNE MICE INFECTED WITH FRANCISELLA TULARENSIS LVS - RAPID EXPRESSION OF MRNA FOR IFN-γ AND TNF-α IN IMMUNE MICE

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Introduction: A memory immune response to infection with the vaccine strain Francisella tularensis LVS is characterized by rapid control and subsequent cradication of bacterial doses that are normally lethal. In the present investigation bacterial viable counts, induction and quantification of cytokine mRNA in foci of infection after a intradermal reinfection were analyzed to further characterize the memory immune response.

Material and Methods: Female Balb/c mice approximately 8 weeks of age were used in the experiments. Mice were challenged with an intradermal (i.d.) inoculation of 3.8 x 10^5 *F. tularensis* LVS and killed by decapitation after 1, 3, 5, 8, and 15 days of infection. One cm^2 of the skin, at the site of inoculation, spleens, livers and regional lymphnodes were homogenized and the number of *F. tularensis* LVS was calculated by plating 10-fold serial dilutions. Mice that received a secondary challenge had been given an subcutaneous inoculation of 1 x 10^4 CFU of *F. tularensis* LVS 5 weeks earlier.

For mRNA preparation and cDNA synthesis, mice were i.d. inoculated with 3 x 10^5 F. tularensis LVS and killed 1, 6, 12, 24, 48, and 72 h later. RNA was isolated from the skin and lymph nodes using a guanidine isothiocyanate-phenol-chloroform single step method, cDNA synthesis folowed by PCR with primers for the cytokines IL-1β, IL-2, IL-2R, IL-4, IL-6, IL-10, IL-12p40, iNOS, TGF-β and GM-CSF. To quantify the amounts of mRNA for TNF-α and IFN-γ a competitive PCR (Clontech, USA) were used.

Results: After primary infection, bacterial numbers increased during the first two days at the inoculation site and then started to decline. A few bacteria were still detected on day 14. By contrast, bacterial numbers declined within one day of infection at the inoculation site of immune mice and all bacteria were eradicated within 8 days. In draining lymph nodes, significantly lower bacterial numbers were detected in immune throughout the course of infection. In immune mice, maximum bacterial numbers were found on day 1, after which infection was brought under control and resolved by day 8, whereas *F. tularensis* organisms were detected until day 14 of infection in non-immune mice.

Some cytokine mRNAs appeared in a somewhat random fashion in the two groups, notably IL-1β, IL-6, IL-15 and GM-CSF. No visible PCR amplicons were observed for IL-2, IL-2R, IL-4 and TGF-β, IL-12p40, INF-α, INF-γ, and iNOS mRNA were observed at 24 h and on after reinfection, but only at 48 and 96 h after primary infection. IL-10 was observed from 12 h and on after reinfection and at 48 and 96 h after primary infection. Background levels were much more prominent in lymph nodes. In some instances the constitutive expression of certain cytokine mRNA hindered assessment of the kinetics. IFN-γ were constitutively present in lymph nodes. IFN-γ mRNA was increased in lymph nodes within 24 h in immune mice. TNF-α and IFN-γ mRNAs were expressed at 5 to 40-fold higher levels 24 h post infection in immune mice when compared with naive mice. Similar differences were observed in repeated experiments.

Conclusion: Presence of greater amounts IFN-γ and TNF-α early at the local site of infection might in itself be sufficient for challenge inocula to be more efficiently contained and eradicated.