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I. ORAL PRESENTATION

IXODID TICKS AS VECTORS OF FRANCISELLA TULARENSIS

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Introduction: An enzootic area of tularemia has been known in the district of Breclav, South Moravia (Czech Republic) since 1936. The role of local vertebrate hosts for F. *tularensis* play the European hare *(Lepus europaeus)* and rodents, and ixodid ticks serve as vectors. We have compared the prevalence of F. *tularensis* among ixodid ticks in the Breclav district with that in some other districts of the Czech and Slovak Republics.

Materials and Methods: Host-seeking ticks were sampled by dragging white flannel flags over vegetation, while a very low proportion (<1%) of arthropods were collected on vertebrates. The ticks were identified, pooled (10 to 20 adults, 25 to 50 nymphs per pool), homogenized in Eagle's MEM or phosphate buffered saline pH7 (PBS), centrifuged, and inoculated in SPF mice. Between 1976 and 1990, the suspensions contained penicillin (200 i.u./ml) and streptomycin (l00,μg/ml) and were inoculated in suckling mice intracerebrally (isolation experiments for arboviruses), whereas since 1991 the homogenates have not contained any antibiotics and have been inoculated in 4 to 6-wk old mice subcutaneously. The mice were observed for 10 days; dead animals were sectioned, and impression smears from the spleen, liver and heart blood were stained by Giemsa's stain. Pure cultures of F. *tularensis* were obtained from the spleen homogenates by plating out on a thioglycollate-glucose-blood agar (TGBA, after Lukáš & Libich). For identification, agglutinating antigens were prepared in saline from 3-d old cultures grown on TGBA plates and tested against diagnostic rabbit sera to F. *tularensis* and *Brucella abortus* (BIOVETA Ivanovice). For titration, the original tick suspensions containing F. *tularensis,* stored at -60°C, were rapidly thawed, diluted serially in PBS and inoculated subcutaneously in 4-wk old mice.

Results: A total *of 33347* ticks in 1487 pools were examined in the districts of Breclav, Znojmo, Vyskov, Český Krumlov, Ceská Lípa and Rožňava during the years 1976 to 1997: 26688 *Ixodes ricinus, 3534 Dermacentor reticulatus,* 2057 *D. marginatus,* 831 *Haemaphysalis punctata* and 327 H. *concinna.* Fifty-five isolates of F. *tularensis* were recovered, and the minimum infection rate was 0.01% in I. *ricinus,* 1.47% in D. *reticulatus* and 0.05% in D. marginatus. The estimates of the F. *tularensis* cells per infected tick ranged from 40 to 69300. Longitudinal studies have been carried out in a natural microfocus (a flood-plain forest ecosystem) of tularemia near Lanžhot, district of Břeclav. In spring of 1995,1996 and 1997, the infection rates of randomly sampled 500 local adult D. *reticulatus* were 2.6% 2.0% and 1.4 %, respectively.

Conclusion: *Dermacentor reticulatus* ticks often harbour F. *tularensis* in the endemic area of tularemia in South Moravia. We found an average of 1.8% of adult D. *reticulatus* infected. This tick species obviously acts as principal vector for, and possibly main reservoir of tularemia in the enzootic focus. Monitoring of adult D. *reticulatus* for F. *tularensis* thus seems to be a very efficient approach in the surveillance of tularemia in the flood-plain forest ecosystem of Eurasia.

DYNAMICS OF INFECTION WITH FRANCISELLA TULARENSIS AND BORRELIA BURGDORFERI S.L. IN RESERVOIR ANIMALS AND TICKS - MARCH RIVER BASIN, AUSTRIA, 1994/1995

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Introduction: As a part of a wider systematic study of reservoirs and vectors of Lyme disease and other zoonoses, one year's followup was conducted in Hohenau in March in the Austrian, Slovakian, and Bohemian borderland, region endemic for tularemia.

Materials and Methods: Organs of live-trapped animals were examined by cultivation and by PCR for the presence of borreliae, in expe-riments on white mice and by cultivation for Fran-cisella tularensis. In ticks collected by flagging borreliae were detected by dark field microscopy, F. tularensis by inoculation of pools into white mice, resp. individually by cultivation.

Results: From a total of 423 animals, infection with Borrelia burgdorferi as well as with F. tularensis was revealed in Apodemus species (A.flavicollis and A. sylvaticus), Clethrionomys glareolus and Microtus arvalis. The dynamics of infection with the two agents differed in time and according to animal species. Borreliae were recorded from May up till January, while F. tularensis from August till December, when an epizootic of tularemia was cought. In ticks, borrelial infection, found only in Ixodes ricinus, peaked in June (21%), reappeared in October (6,3%) and April (5%), while F. tularensis was isolated repeatedly only from Dermacentor reticulatus, beginning in October (3,5%), to April.

Conclucions: Our findings indicate, that in regions with occurrance of tularemia, the risk of infection with borreliae could be modified mainly during epizootic outbreaks and show the different role of two tick species involved in the transmission.

EPIDEMIOLOGICAL ANALYSIS OF TULARENMIA IN SLOVAKIA

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Introduction: The territory of Western Slovakia has been known as an endemic area of tularemia for several decades. First cases in humans were recorded in 1936. After a large epidemic in the 1960s, this area was subjected to epidemiological surveillance, which revealed the existence of different types of natural foci and a wide range of reservoir animals and vectors *of F. tularensis.*

Materials and Methods: Cases of human tularemia recorded in Slovakia over the years 1950 - 1996 were analysed according to their basic epidemiological characteristics.With the aim *to assess* chanes in the epidemiology of the disease, comparison of data from Western Slovakia was made in ten-years periods (1961-1996).

Results: From a total of 3705 cases of tularemia recorded in Slovakia since 1950, 84% have occurred in its western region. The average annual morbidity declined from 5.5 per 100 000 population in the 1960s to 0.3 during a period of sporadic occurrence of the disease in 1980-1994. As a result of an epidemic, the average morbidity rate in Western Slovakia increased 7-times in 1995-1996 comparing with the interepidemic period. Analysis of the incidence of tularemia in endemic region (1961-1996) showed the important changes noccerning mainly the geographical distribution, seasonal occurrance and clinical manifestations with respect to different sources of infection and routes of transmission.

Conclusions: Decrease of morbidity from tularemia observed in Slovakia since the epidemic in the 1960s has been apparently due to the lower exposition of population, resulting from changes in technology of agricultural production, limited hunting as well as vaccination, mainly of professionally exposed groups. Maintenance of active natural foci in the endemic area is drawing attention to the need of systematic surveillance for epidemiologic prognosing and effective prevention.

TULAREMIA IN BULGARIA

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Introduction: In this material we present and characterise an "Endemic focus" of tularemia in north-east region of Bulgaria. That is for the first time described in 1962 by our group.

Materials and methods: We utilised a complex of epidemiological, microbiological, immunological, biological, clinical diagnostic etc.methods, which are conventional for this type of investigation.

Results: A strain with high virulence of Francicella tularensis-palearctica was isolated named from us F.tularensis-Srebarna19. This strain was found in Ondatra zibetica. The animal populations inhabit biotop a lake not far from Danube river. Other biology spities from the same population were analysed. It was described pulmonary form of the disease in one patient. In skin test human population investigation the allergy status was positive in 8-10% of citizens in the area.

Next investigation was made in 1972. During the period 62-72 no dynamic changes were observed. New foci were not founded.

Discussion: Mechanisms of burst of the focus and its dynamic were discussed.

THE METHODICAL APPROACHES TO DESIGNING OF RECOMBINANT LIVE VACCINE *FRANCISELLA TULARENSIS* STRAINES.

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Introduction: *F. tularensis is* one of the several intracellular parasites for which live vaccine was created. This vaccine induces development of strong and prolonged cellular and humoral immunity. An idea seems attractive of employment of F. *tularensis* strains 15/10 or LVS as a base of live recombinant vaccines against different deases.To solve this problem one have to develope delivery and stable expression system for foreign protective antigens into the tularemia microbe, as far as to modify vaccine strain, namely one have to make immunogenic properties of the vaccine strain more stable and to reduce residual virulence for mice.The purpose of present study was choice of the optimal method of plasmid DNA transformation in *F. tularensis,* development of recombinant *F. tularensis* strains without additional antibiotic resistance markers and studijing recipient properties of the recA-like variant of F. *tularensis* 15/10.

Materials ans Methods: *F.tularensis* 15/10 strain (collection of SRCAM, Obolensk); recA-like strain [1], pFNL10, pFNL2OO, pTV24 plasmids [2], pFPA14 plasmid encoding protective antigen *of Bacillis anthracis* [3] were used in this study. Cultivation of bacteria, preparation of plasmid DNA transformation of bacteria by plasmid DNA and construction of recombinant plasmids were carried out as described earlier [2]. Cryotransformation of the plasmid DNA in F. *tularensis* was carried out

as in the work [2], plasmid DNA electroporation into the *F. tularensis* cells was carried out for cell suspension (10^{10} CFU/ml), electric field intensity 12,5 kV/cm, in a 2,5 msec. pulse regime. Electroporation buffer contained 0.5 M sacrose,1 mM EDTA, pH 7.5.

Results: A comparison of cryotransformation and electroporation method efficacy of plasmid DNA introduction into *F. tularensis* 15/10 vaccine strain showed, that both methods provide nearly the same transformation frequency, namely about 10^6 clones per 1 mkg of plasmid DNA. Clones on selective media appeared in 48-72 hours at 37°C in both cases.

Recombinant plasmid carrying *F. tularensis* strain without antibiotic resistance markers was developed by co transformation of the recombinant plasmid and pTV24 plasmid which was competent cell marker. Recombinant plasmid without antibiotic resistance gene was constructed as it described below. Co transformation was cárried out with mixture of the 0,2 mkg of Bgl II-ClaI fragments of pFNL10 plasmid and BamHI-fragment of pFPA14 plasmid ligated by T4 DNA lygase, and the 0.001 mkg of pTV24 plasmid DNA. Transformant selection was performed using tetracyclin-containing medium, and selection of transformant clones producing protective antigen of B. *anthracis* was carried out by immunoblot method using specific serum. pTV24 plasmid disappeared in a case of biplasmid *F. tularensis* strain growth in a liquid medium.

To reduce recombination events frequency in F. *tularensis,* the rec A-like variant was used. Plasmid transformation was performed in recA-like variant of F. *tularensis* 15/10 and its biological properties were studied.

Conclusions: Optimal condition for cryetransformation and electroporation are proposéd for plasmid DNA transformation into *F. tularensis.* Recombinant *F. tularensis* strain witout additional antibiotic resistance markers was obtained. RecA-like variant of vaccine *F. tularensis* strain can be used for recombinant strains construction.

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FLOW CYTOMETRIC ASSESSMENT OF THE SURVIVAL RATIO OF *FRANCISELLA TULARENSIS* IN AEROBIOLOGICAL SAMPLES.

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Survival of microorganisms in aerobiological samples is often assessed by a survival ratio (SR), which is the ratio between the viable or metabolic active number (MA) of microorganisms to the total number (TOT) of microorganisms. A method to determine survival ratios with flow cytometry was developed for *Francisella tularensis,* the causative agent of tularemia. F. *tularensis is* a fastidious bacteria that can be transmitted by aerosol and constitute as such a valid model organism for aerobiological studies. The total number of F. *tularensis* cells was determined by specific targeting with monoclonal antibodies and detected by phycoerytrine (PE) conjugated secondary antibodies. The metabolic active part of the targeted F. *tularensis* cells was quantified by staining with rhodamine 123 (Rh123). Application of the presented method showed higher precision compared to an earlier developed method for survival ratios, achieved with plate count (VC) and Coulter Counter (CC) measurements. The coefficient of variation between samples for the new method was below 5 % for the survival ratio. Comparison of VC yield with MA yield showed consistently higher values for MA. The survival ratios of F. *tularensis* in samples taken before and after aerosolisation were analysed. SR for F. *tularensis* determined with the new method decreased approximately 19 % whereas SR determined with VC and CC decreased 55 % after passage through aerosol state.

MOLECULAR CHARACTERISATION OF *A FRANCISELLA TULARENSIS* CLONING VECTOR

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Introduction: Studies on bacterial properties contributing to virulence *of Francisella tularensis* have so far been limited. One explanation for this is the lack of a genetic system for the bacterium, enabling construction of genetically defined mutants. During the last years, cloning vectors able to replicate in F. *tularensis* have been described. One of these vectors is pKK202, originating from plasmid pFNL2OO. Plasmid pFNL2OO is a hybrid plasmid based on the 4 kilobase cryptic plasmid pFNL10, originally isolated from the *Francisella novicida* like strain F6186, and the *Escherichia coli* plasmid vector pBR328. The plasmid expresses

resistance in F. *tularensis to* tetracycline and chloramphenicol due to the presence of the resistance genes from pBR328. Plasmid pKK202 harbours the p15A origin of replication and can therefore, in addition to pFNL2OO, also replicate in E. *coli.*

The presence of the E. *coli* tetracycline gene (tet-gene) in F. *tularensis* normally does not result in resistance. However, the original construction of pFNL2OO resulted in a plasmid which expresses tetracycline resistance in *F. tularensis.*

The purpose of the present study was to further characterise the promoter region of the tet-gene in plasmid pKK202 and also to preliminary characterise the F *tularensis* replication origin of the plasmid.

Material and Methods: Sequence analysis of a 1.9 kilobase fragment was carried out by "plasmid walking" using oligonucleotides as primers.

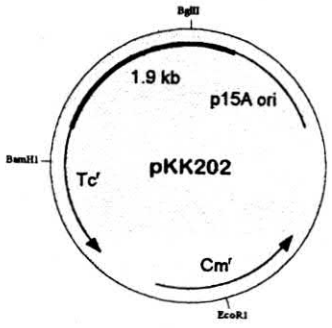
The transcriptional initiation start point of the tet-gene was determined by primer extension.

The study of a point mutation in the promoter region of the tet-gene in pKK202 was accomplished by exchanging a 300 base pair fragment from plasmid pACYC184 with the corresponding fragment of pKK202. The preliminary characterisation of the F. *tularensis* replication origin of plasmid pKK202 was performed by deletion studies using the polymerase chain reaction.

Results: A 1.9 kilobase fragment from pKK202, located between the tet-gene and the p15A origin of replication, was sequenced (see figure). The sequence revealed a base shift, from a G to a T, in the identified promoter region for the tet-gene of plasmid pBR328. The base shift was in the E. *coli* transcriptional start site for the tet-gene in the original plasmid pBR328.

Primer extension, to identify the transcriptional start site for the tet-gene of plasmid pKK202, was performed. It was found that the transcriptional start site was located 180 base pair upstream of the original transcriptional start site of the tet-gene. In E. coli the transcriptional start'site for the tet-gene in pKK202 was the same as in F. *tularensis.*

To check the importance for the base pair shift, from G to T, a shift back to a G was performed. It was found that the tetracycline resistance was not expressed in *F. tularensis* when the T in pKK202 was replaced with a G. In E. *coli,* the tetracycline resistance was still expressed after the shift back.



The F. *tularensis* replication origin of pKK202 was found to be located within the 1.9 kilobase fragment. This was initially shown by introducing a DNA fragment into the unique Bg1II restriction endonuclease site of the plasmid, which resulted in that pKK202 no longer could replicate in F. *tularensis.* The results of a further mapping of the *F. tularensis* replication origin is also reported.

CHARACTERIZATION OF LIPOPOLYSACCHARIDES IN DIFFERENT SPECIES OF FRANCISELLA

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Lipopolysaccharide (LPS) is important cellular component determing toxic, virulent and immunological properties of Gram-negative bacteria. However, biological function of Francisella tularensis LPS is not clear because it has no toxic and pyrogenic activities. There is no data about structure and function of LPS of new representatives of Francisella genus - F. novicida-like and F. philomiragia. In the present study we investigated some biological properties of LPS in different species of Francisella.

The comparative analysis of LPS preparations isolated from bacteria by method of Darveau and Hancock showed that most Francisella representatives (F. tularensis, F.novicida, F.novicida-like) had S-LPS, visualized in silver-stained PAAG as a <<ladder>> typical of Gram-negative bacteria. However, the <<ladder>> of F.philomiragia S-LPS had two dominant components with definite O-chain length. F. tularensis isogenic avirulent variants possessed either R-LPS or defective S-LPS. Only F.tularensis S-LPS had species-specific antigenic activity in immunoprecipitation, whereas R-LPS of F. tularensis and S-LPS of other Francisella species had common nonspecific antigens. In neutralization reaction of specific antibodies against tularemia agent S-LPS preparations of F. tularensis were positive over concentration range 170 - 700 ng, whereas R-LPS of F. tularensis and S-LPS of other Francisella species - only over the concentration range 6 - 500 mcg. LPS preparations of all Francisella representatives had no toxic and immunogenic properties against tularemia agent, injected 21 days after immunization. At the same time S-LPS of F. tularensis and of F. philomiragia which were injected to ' mice 72 h before challenge with virulent F. tularensis strain (100 DCL and 1000 DCL) were able to increase average animal life and to protect part of animals against lethal infection (17-33 % of survived mice). This results allowed to suppose that S-LPS of F.

tularensis and of F.philomiragia had immunomodulating activity, as opposed to R-LPS of F. tularensis and S-LPS of F.novicida and F. novicida-like. The study of anti-toxic activity of Francisella LPS revealed that R-LPS of F.tularensis and other Francisella species were characterized with tendency to protect the animals against lethal doses of endotoxin of Salmonella typhimurium. S-LPS of all Francisella species had not this activity.

Thus, the results of present investigation showed that LPS from various species of Francisella differed from each other by immunological, immunomodulating and antitoxic activities.

QUANTIFICATION *OF FRANCISELLA TULARENSIS BY* SPECIFIC CAPTURE ELISA AND BY FLOWCYTOMETRY

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Introduction: *Francisella tularensis (F. tularensis)* causing tularemia represents an interesting model to study the immunopathology of facultative intracellular bacteria. The understanding of these mechanisms is important for the diagnosis, therapy and vaccine improvement of tularemia. Up to now the detection and quantification of F. *tularensis* is mainly achieved by cultivation on cystein-containing selective agars which is time and work consuming. Recently, detection methods based on polymerase chain reaction have been described. However, especially for in-vitro experiments, but also for diagnostic purposes, the quantification of bacteria in relatively large sample numbers and the discriminaton between live and dead bacteria is of interest. Thus, the aim of this study was (i) the development of a F. -tularensis-specific capture enzymeimmunoassay (EIA) based on monoclonal antibodies (mAb) and (ii) the evaluation of a live staining of F *tularensis* with subsequent detection by flowcytometry.

Materials and Methods: Hyperimmune sera from rabbits were obtained by repeated immunization i.m. of animals with inactivated bacteria from an avirulent strain of F. *tularensis* (ATCC 6223). A panel of mono-clonal antibodies (mAb) developed and provided by Dr. Greiser-Wilke (Institute of Virology, Hannover Veterinary School, Germany) was screened by solid phase EIA, immunofluorescence (IF) and Westernblot (WB) using the F. *tularensis* reference strain. Further isolates of F. *tularensis* from men and hares were kindly provided by Dr. P. Berdal (Norwegian Defence Microbiological Laboratory, Oslo, Norway) and Dr. E. Hofer (Bundesanstalt für Tierseuchenbekämpfung, Mödling, Austria). Other used bacterial antigens were isolated and characterized by Dr. Niederwöhrmeier (Wissenschaftliches Institut für Schutztechnologien, ABC Schutz, Munster, Germany). For capture EIA,10 μg of a selected mAb (FT-27) was coated to microtitre plates. Bacteria were added at different concentrations and incubated for at least 2 hours. The bound antigen was detected either by a specific rabbit serum and anti-rabbit-Ig-peroxidase (POD) or by the biotin labeled mAb (FT-27-b) and Streptavidin-POD. For flowcytometry, the bacteria were stained with Live/Dead BacLight (Molecular Probes) according to the supplied assay description or with FITC-labelled mAb (FT-27- FITC). The cells were analyzed by an EPICS XL (Coulter Electronics) creating a live and dead region in a site scatter-FLI (green) dot blot histogram. For comparison, the number of bacteria was determined by counting the colony forming units (CFU) from serial dilutions on cystein-heart-agar supplemented with 10% sheep blood (Difco) and photometrically at wave length 560 nm.

Results: A panel of 11 murine mAbs and 2 rabbit hyperimmune sera raised against inactivated F. *tularensis* (ATCC 6223) showed a specific reactivity in solid-phase EIAs and IF. WB studies revealed that all mAbs recognized the lipopolysaccharid of bacteria. In addition, all mAbs and rabbit sera reacted with all tested 24 human and hare isolates of F. *tularensis* in solid phase EIA and IF. In contrast, no cross reactivity was observed with various isolates *of Yersinia* spp., *Brucella spp, and Burkholderia* spp.. From dilution curves in solid phase EIA, a panel of four mAbs showed the highest reactivity. These mAbs were tested as capture antibodies by binding 10 μg/ml to solid phase. One mAb (Ft-27) was selected by the best capacity to bind bacteria (10^4bacteria/ 100 μl) when used a rabbit serum as detecting antibody. In a further step, the mAb Ft-27 was labeled with biotin and used as detecting antibody in capture EIA. The same mAb as capture (10 μg/ml) and detecting antibody (5 μg/ml) achieved a sensitivity of 10^4-10^3 bacteria/100 μl. The assay will now be evaluated for the detection of F. *tularensis* in various biological specimens.

In addition, the F. *tularensis* was detected by flowcytometry. Using the BacLight staining reagents (Syto 9/ propidium iodide) live and dead bacteria could be discriminated in suspensions containing different proportions of propanol-killed and non-killed bacteria. Analyzing a defined volume of the bacterial suspension by "stop and volume" modus of the flowcytometer, quantitative data of bacteria were obtained. These results correlated significantly with the optical density at 560 nm of the bacterial suspension and the number of bacteria determined by CFU.

Conclusion: A specific EIA has been developed that is principally suitable for clinical diagnostic as well as for the detection *of Francisella tularensis in*

in-vitro-experiments. The flowcytometric analysis *of Francisella tularensis* represents a rapid method for counting and discrimination of live and dead bacteria. Attempts are underway to stain the bacteria with specific antibodies for extra- and intracellular detection and quantification.

CLINICAL EXPRESSION OF TULAREMIA

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Since 1931, when tularemia was first recognized in Sweden, the annual incidence has varied widely. Ulceroglandular and respiratory tularemia have been the predominant forms of the disease observed. The clinical expression of these forms will be described. Moreover, a few cases of oropharyngeal tularemia septicemia, and tularemia meningitis will be reviewed. In routine clinical work, the diagnosis of tularemia is reached mainly by serological investigation. Culture of specimens requires safety conditions and vaccinated personnel. Promising results have recently been obtained by use of a PCR technique developed for skin samples from patients with ulceroglandular tularemia. Aspects on diagnostic methods, therapy, complications, and outcome of tularemia will be given.

LESSONS FROM *FRANCISELLA*: THE CONTINUUM OF THE IMMUNE RESPONSE TO INTRACELLULAR INFECTION.

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We have investigated the mechanisms of initial resistance to infection and generation of protective immunity to intracellular bacteria, using *Francisella tularensis* LVS infection of mice as a model. Initial resistance to infection, which lasts about 20 - 30 days, depends on the activity of neutrophils, macrophages and natural killer cells, through production of tumor necrosis factor α , interferon γ, and Interleukin 12. A second, month long phase of innate immunity that is lymphocyte dependent has also been characterized in murine responses to *Francisella*: very strong specific protective immunity to lethal LVS challenge develops in both normal and nu/nu mice, but not scid mice, within 2 - 3 days after sublethal intradermal priming with LVS. In vivo depletion studies, transfer studies, and studies using knockout mice suggest a major role in this protection for B 220^+ B cells that is independent of antibody production. This response also operates in *Listeria monocytogenes* infection, and is nonspecific. Finally, experiments using either mice depleted of $CD4^+$, $CD8^+$, or $\gamma\delta^+$ T cells, or scid mice reconstituted with various T cell subpopulations, demonstrate that loss of either $CD4^+$ or $CD8^+$T cells does not affect generation and expression of long term (late phase) specific protective immunity to LVS, however, loss of $\gamma\delta^+$ T cells has a minor effect on the strength of protection. Moreover, B cell knockout mice are seriously impaired in their ability to survive secondary lethal challenge. Again, this defect does not seem to be due to absence of antibody production. The bacterial antigens responsible for induction of early protection are under intense study. Intramuscular inoculation of mice with chromosomal LVS DNA, as well with oligonucleotide DNA containing unmethylated CpG motifs, results in the generation of strong protective immunity to both early and late lethal LVS challenges, LVS chromosomal DNA stimulates production of IFN γ , IL12, and IL6 *in vitro*. Studies using various immunodeficient and knockout mice revealed that DNA-stimulated protection is highly dependent on lymphocytes, especially B cells. Since both innate and specific protective mechanisms involve Th1-like cytokine production, it is likely that each phase of the innate and specific immune response follows, and is dependent on, successful induction of the previous phase.

IDENTIFICATION OF PROTEINS OF *FRANCISELLA TULARENSIS* INDUCED DURING GROWTH IN MACROPHAGES AND CLONING OF A PROMINENTLY INDUCED 23-KDA PROTEIN.

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Introduction: The adaptation of facultative intracellular bacteria to host macrophages involves regulation of the synthesis of bacterial proteins. We comparatively analyzed the protein synthesis of *Francisella tularensis* LVS growing intracellularly in the macrophage-like murine cell line J774 and extracellularly in culture medium.

Materials and Methods: We here studied the translational response of F. *tularensis* LVS during growth within the murine macrophage-like cell line J774 by two-dimensional gel electrophoresis. After pulse-labeling with (^{35}S]-methionine and separation by gel electrophoresis, pattern of a few proteins induced was compared. All DNA manipulations, including purification, digestion with restriction endonucleases, ligations, and gel electrophoresis, were performed according to the standard protocols or according to the manufacturer's instructions.

Results: Induction of a few proteins was demonstrated during intracellular growth. One of them, a 23-kDa protein was prominently induced in the macrophages and also when extracellularly growing F. *tularensis* was exposed to hydrogen peroxide. After isolation of the 23-kDa protein from a preparative two-dimensional gel, an N-terminal peptide and two peptides obtained by trypsin digestion were sequenced. Based on the sequences, degenerate oligonucleotides were constructed for use as primers in a polymerase chain reaction. Hybridization of amplified DNA to Xbal-digested LVS DNA identified the gene of the 23-kDa protein in a 1.3-kilobase (kb) DNA fragment. Nucleotide sequence analysis revealed an open reading frame encoding a putative protein of a calculated molecular weight of 22.2 kDa. The open reading frame was preceded by a sequence typical of ribosomal binding sites in *Escherichia coli*. The amplified gene was successfully expressed by the pTrc99A vector in E. coli under control of the trc promoter. The gene product showed the same mobility and immunoreactivity as the 23-kDa protein of F. *tularensis*. The deduced amino acid sequence showed no significant homology with protein sequences of current databanks.

Conclusion: A 23-kDa protein of F. *tularensis* LVS was found to be up-regulated to a considerable extent during growth in macrophages and in response to hydrogen peroxide exposure. The function of the protein is so far elusive.

TULAREMIA, BUT NOT TULAREMIA VACCINATION, RESULTS IN EXPANSION OF Vγ9Vδ2 CELLS.

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Introduction: Tularemia is caused by the facultative intracellular bacterium *Francisella tularensis. A* previous report describing an increase of Vγ9Vδ2 T cells in a tularemia patient prompted us to investigate whether this is a general phenomenon during tularemia and also to determine whether phosphoantigens, powerful stimuli for Vγ9Vδ2 cells, are present in F. *tularensis.*

Materials and Methods: Through general practitioners at various locations in northern Sweden and Finland, blood samples were obtained from patients with suspected ulceroglandular tularemia. All cases were subsequently confirmed as tularemia by serology. Flow cytofluorometric analysis was carried out on 50 μ1 of whole blood from each individual. For analysis, lymphocytes were gated according to their morphological parameters and analyzed with the Cell Quest software (Becton-Dickinson). Results were expressed as percentage of lymphocytes, or as percentage of $CD3^+$ cells staining positively for a given label.

For preparation *of Francisella* extracts, a previously described method for preparation of phosphoantigens *of Mycobacterium tuberculosis* was followed.

Results: In samples drawn from 13 patients 7-18 days after onset of tularemia, Vγ9Vδ2 represented on average 30.5% of the $CD3^+$ cells, and nearly 100% of $γδ^+$ T-cells. In contrast, no significant increase was observed after vaccination with after vaccination with F. *tularensis* LVS, and the number of Vγ9Vδ2 cells constituted on average 5.3%, as in control subjects. Phosphoantigenic activity was detected in extracts from a clinical isolate (FSC171) and from LVS. The F. *tularensis* phosphoantigens triggered in vitro both proliferative responses of polyclonal human Vγ9Vδ2 PBL and cytotoxic responses or TNF release of selected Vγ9Vδ2 T cell clones. When compared to that of the clinical isolate, the phosphoantigenic activity was found to be lower in the LVS strain.

Conclusion: A significant increase of Vγ9Vδ2 cells occurs in peripheral blood of tularemia patients. The higher phosphoantigen load of the clinical isolate and the massive increase of Vγ9Vδ2 cells in peripheral blood of tularemia patients, but not of vaccinees, indicate that F. *tularensis* phosphoantigens may be responsible for the observed increase of Vγ9Vδ2 cells during tularemia.

STUDIES OF FRANCISELLA TULARENSIS 63 KDA PROTEIN BINDING SITES ON HUMAN IGG

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Introduction: A 63 kDa protein has been isolated from the vaccine strain 15 of *Francisella tularensis* (type B) able to interact with human and rabbit immunoglobulins obtained from non-immune sera. The p63 gene was cloned and partly sequenced. It has been found that the sequence is identical to the independently cloned and sequenced GroEL homolog (molecular chaperone Hsp 65 of F. *tularensis*. The amino acid composition of the natural p63 and the amino acid composition of GroEL homolog are compared. The comparison of other characteristics of p63 and GroEL allow to conclude that p63 is GroEL homolog abnormally expressed on the surface of F. tularensis.

Materials and Methods: Affinity chromatography (affine sorbent p63-Sepharose 4B) has been used to isolate a human IgG. The obtained human IgG is analysed by means of SDS-PAGE and immunoprecipitacion with antisera to IgG subclasses and to κ- and λ- light chains. IgG fragments separated in SDS-PAGE are transferred onto a nitrocellulose membrane and detected using p63 labeled by horse-radish peroxidase.

Results: The human IgG isolated by affinity chromatography is detected as a IgG_1 subclass with both κ- and λ- chains. Intact molecules of human IgG have two binding sites for the 63 kDa protein *F. tularensis on* Fab- and Fc- fragments. Effective binding of the protein with L- and H-chains of IgG has been observed. Furthermore, quite unexpectedly we have found that the bindings of the p63 protein with trypsinogen and lysozyme are experimentaly used as the marker proteins of molecular mass. Counlusion. Interaction of p63 with H- chains of Fc- fragment (CH_2-CH_3 domens) may testify that this binding is defined as the highly conserved amino acid sequense. Interaction of p63 with $F(ab')_2$-, Fab-fragments of IgG can be explained by the presence of cross-reacted antibodies to stress proteins (Hsp 65) of bacteria. Further studies of the 63 kDa protein can contribute to the understanding of molecular mechanisms of an evasion *of F. tularensis* from adequate immune recognition and subversion by the bacterium of the macrophage / monocyte response.

TEMPERATURE SENSITIVE FUNCTIONS RELEVANT FOR THE *FRANCISELLA* PATHOGENICITY: HEATSHOCK CATALASE FUNCTION.

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Introduction: Heat shock and oxidation stress are among the main factors potentially involved in regulation of bacterial pathogenicity functions, especially for *Francisella tularensis* that deals with high temperature of host rodents and has to defend itself against oxidative activity of macrophages and other cells of immunity response in course of tularemia infection[1,2,3]. Mutants in our genetic collection *of Francisella tularensis* strains deficient for pathogenicity and induction of cellular protective immunity in the host organisms were divided for two major classes:(1) the mutants having changes in the cell wall architecture attributed to membrane proteins and (2) the mutants affecting heat response functions restored by supplementation of blood or hemoglobin added to cultivation medium[4,5]. The second class of mutants, having ultimately impaired virulence and immunogenicity, suggested a possible involvement of the mutations in the genes of heat shock and oxidative stress. This notion seems reasonable, if one considers the activity of catalases, including temperature sensitive ones, in dealing with oxidative stress of prokaryotic and eukaryotic cells relevant for cellular adaptation[6]. In attempts to elucidate the possible role of heat shock and oxidative stress functions in the mechanisms *of Francisella tularensis* pathogenicity we have chosen to characterize the catalase functioning in the virulent bacteria under the different conditions of bacterial incubation and compare it with the one in the nonvirulent mutants and related *Francisella* species unable to cause tularemia.

Materials and Methods: Francisella *tularensis* type A and B virulent strains, and their sds^S-ery^R or t^R mutants deficient in virulence and pathogenicity have been used for testing catalase activity, as well *as Francisella novicida* and *Francisella philomiragia* strains. The bacterial cells were grown on the egg yolk or LA-cysteine medium supplemented with hemoglobin. The cultures were incubated for 96 hours at the temperatures 30°C, 37°C or 42°C. The catalase activity has been tested by a semiquantitative technique in modification[7].

Results: Functional activity of catalase was registered for virulent cells *of Francisella tularensis* types A and B grown at both nutritional media used at 30°C and 37°C. The slight increase in activity should be noted for the strains growing on the hemoglobin containing medium. Incubation of the

same strains at 42°C, the optimal heat shock temperature, has resulted in decrease of the registered catalase activity for type B virulent strains for half an order of magnitude on both incubation media used. Type A strains grow only on hemoglobin supplemented medium at this temperature demonstrating the decreased but reproducible level of activity comparable with the level of catalase activity in type B cells.

The catalase activity in t^R mutants of type A is diminished to the minimal level detectable by the technique used on both nutritional media at all temperatures tested. In contrast, the sds^R-ery^S demonstrate the catalase activity comparable to the one peculiar for the wild type bacteria.

The catalase activity *in Francisella novicida* is comparable in properties to the activity revealed by *Francisella tularensis* cells. The unexpected finding of this work is the high level catalase activity demonstrated by *Francisella philomiragia* cells. It exceeded the activity *in Francisella tularensis* and *Francisella novicida* cells about fivefold, showing no visible dependence on hemoglobin in cultivation media at 30°C and 37°C. At 42°C the catalase activity was ultimately diminished under all experimental conditions tested.

Conclusion: The functioning of catalase activity *in Francisella tularensis* cells of type A and B is comparable and stimulated by the presence of hemoglobin in media used for bacterial cultivation. It is less efficient at the optional heat shock temperature - 42°C. The diminished catalase activity in a nonvirulent heat shock mutant *of Francisella tularensis* suggests the involvement of the activity in type A cells temperature and hemoglobin dependent expression of virulence.

The similarity between the medium level catalase activities *in Francisella tularensis* and *Francisella novicida* is in contrast to the high level activity *in Francisella philomiragia,* ultimately heat shock sensitive and independent of hemoglobin. This difference permits further molecular genetic study of the evolvement of catalase coding genes and their functions in the evolution *of Francisella* generum pathogenicity.

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MACROPHAGE PROTEINS INVOLVED IN MECHANISMS OF GENETIC CONTROL OF INNATE RESISTANCE TO INTRACELLULAR PATHOGENS

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Introduction: This study was designed to analyse the association *of Nramp1* and/or *Lps* genes with differential protein expression in macrophages in order to select candidate proteins that might be related to resistance/susceptibility to various microbial infections under the control of the *Nramp1* and/or Lps genes.

Materials and Methods: The macrophage cell lines derived from bone-marrow *of Nramp1 or Lps* congenic mice were utilized and high resolution two-dimensional electrophoresis, separating proteins according to their charge and size, was used as a window into alterations in gene expression and a mean to compare the macrophages carrying resistant allele *of Nramp1* gene and/or normal allele of Lps gene with their counterparts carrying either susceptible allele of *Nramp1* or defective allele *of Lps* gene.

Results: We demonstrate that the changes of constitutive levels of two proteins named according to their pI/Mw p6.6/25 and p7.0/22 discriminate satisfactorily not only the macrophages congenic *at Nramp1* gene but also the macrophages congenic *at Lps* gene, thus reflecting their common genotype ($Nramp1^r$, Lps^n). Furthermore, the decreased constitutive levels of these proteins in macrophages carrying defective allele *of Lps* but preserving resistant allele *of Nramp1* can be, at least in part, restored by stimulation with interferon gamma or lipopolysaccharide. Tentative results identified the p6.6/25 and 7.0/22 proteins as Bcl-2 and manganese superoxidde dismutase, the pro-teins that are important in the regulation of intrace-llular redox balance and the regulation of apoptosis in macrophages.

Conclusion: These protein alterations might reflect closely transport functions of ions or other charged substrates suggested for Nramp 1 protein and for Bcl-2.