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III. REVIVAL OF A RESULTS

RR2 STOCHASTIC MODEL *OF FRANCISELLA TULARENSIS* INFECTION (1976)

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Introduction: The proliferation of microbes in organs of infected individuals is a very complicated dynamic process. In seventies dr. Libich tried to solve this problem using stochastic model utilising general principles of behaviour of very complexed systems.

Material and methods: The basis for the development of stochastic model of *Francisella tularensis* infection in experimental animals was the number of CFU in organs of infected mice and the number of individual microbes in infected cells calculated on the basis of immunofluorescence. The theoretical assumption was that the proliferation of microbes is possible only in a limited number of infected cells. The rate of so-called "resistant" (eliminator) and "sensitive" (replicator) elements is changed during the infection and this rate can be characterized as an "dynamic equilibrium".

Results: The proliferation of microbes is a stepwise process composed of sequence of events: phagocytosis - intracellular replication - killing and disruption of infected cells - spreading of microbes to extracellular environment. The entrance to individual steps can be characterised by probability p that the microbe enter to eliminator (p) or replicator (1- p) element. The function of replicator can be defined as replicative activity $c = 2^x$, where x is the number of intracellular division of microbe. The kinetic of changes in immunoregulatory system can be characterised by k-transformation of proliferation curve according the equation

$$n_t = n_0 \times e^{kt} .$$

Conclusion: The suggested model was tested using experiments with the limitation of proliferation *of Francisella tularensis* I5L microbes by Kanamycin in organs of infected guinea pigs and using *in vitro* model *of Francisella tularensis* 15L infection (see also RR3). The "eliminators" are the donors of regulatory signals for the control of "dynamic equilibrium" in every moments in the course of F. *tularensis* 15L infection.

RR3 IN VITRO MODEL OF FRANCISELLA TULARENSIS INFECTION (1980)

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Introduction: The cells of mononuclear phagocyte system are generally thought to be the third cellular type that participates in almost all phases of immune reactions. Apart from their function in morphogenesis and necrophagocytosis, mononuclear phagocytes mediate harvest, handling and presentation of antigen, production of cytokines, regulation of differentiation pathway of several subpopulations of T helper lymphocytes. After cooperation with T cells, macrophages take part in killing and destruction of transformed cells and microbes.

In late seventies, we try to study the first stages of interaction *of Francisella tularensis* with mononuclear phagocytic cells (primary interaction) using *in vitro* method of flying cover slip cultures of murine peritoneal adherent cells.

Material and Methods: The cultures (more than 95% of macrophages) were prepared from immunized (20 days after s.c. infection of mice with F. *tularensis* strain 15L,1.2 x 10^2 live microbes per mouse) and control mice (without any *in vivo* treatment). The *in vitro* infection was done with *Francisella tularensis* strain 15L and in parralel cultures with F.*tularensis* strain 130 (wild virulent European strain passaged in laboratory condition on guinea pigs and thioglycolate-glucose-blood agar /TGKA/). The multiplication of infection was 1, 10, and 100 microbes per 1 cell of culture, respectively. The number of tularemia microbes in individual cell was calculated after direct immunofluorescent labelling of F. *tularensis* in methanol fixed cover slip cultures.

Results and Discussion: By the comparison of real distribution of microbes in cell cultures with theoretical Poisson's distribution function we can state that the distribution of microbes is a random process. The number of infected mononuclear phagocytes depends on multiplicity of infection *in vitro* and on the time of in *vitro* contact of cells with microbes. When we admit the concept of functional heterogeneity of macrophage population than very close correlation of real distribution with Poisson's distribution function suggests the same "susceptibility" of all cultures used to in *vitro* infection.

On the other hand, the number of infected cells in cultures prepared from control mice decreased only slightly (about 10%) during first two hours of cultivation in comparison with cultures prepared from immunized mice (decrease more than 40%). This result document the property of some part of macrophage population isolated from immunized mice and to a lesser extent from control mice to limit intracellular survival of F. *tularensis* strain 15L and also strain 130. Then the number of infected cell remains stable up to 16 hour of cultivation. During this period the number of microbes per infected cell grown exponentially. From the experimental data obtained from this system we were

able to determine the generation time *of Francisella tularensis* strain 15L and strain 130. The generation time of F. *tularensis* microbes 15L and 130 in adherent peritoneal cells cultivated *in vitro* was calculated to be 4.6 and 4.5 hours, respectively.

First dead cells was observed in cultures 18 hours after *in vitro* infection. The cells die with the same dynamics in cultures prepared from immunized and control mice. After 72 hours of cultivation 85% and 39% of cells were killed in cultures prepared from control and immunized mice, resp.

Conclusions: "We can summarize that there is a group of macrophages within populations of mononuclear phagocytic cells that resist *Francisella tularensis* infection even in control animals. The number of resistant type of macrophages increased after the immunization (primary infection). Resistant part of macrophage population did not express increased phagocytic activity in comparison to sensitive one. The term sensitivity and resistance is used in the sense of resistance to microbes *of Francisella tularensis* under the scope. The infection of macrophages occurs in steps. The duration of one step (period between the infection of mononuclear phagocytic cell and the dead and disruption of cell accompanied with spreading of intracellularly proliferated F. *tularensis)* ranges from 16 to 20 hours. During this time the number of microbes increases in cell from 1 to 32" *(Macela* A.: *CSc. Thesis, Fac. Sci.,* Prague,1980).

RR4 PASSIVE TRANSFER OF IMMUNITY *TO FRANCISELLA TULARENSIS* INFECTION IN IRRADIATED MICE (1983)

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Introduction: Nowadays, the specific antitularemic antibodies are thought to play a minimal role (if any) in the long lasting protection against *Francisella tularensis* LVS. Only IgG isotype(s) are responsible for the passive transfer of limited protection. The role of specific antibodies in early protective immunity seems to be unlikely. However, in spite of the experimental data the presence of circulating specifc antitularemic antibodies is only one of the immune parameters that closely correlate with protection against F. *tularensis* infection for a long period after immunization or primary infection.

Since 1980 to 1985 we have reviewed the possibilites to protect irradiated hosts against intracellular microbial pathogens. Among several models was the model of protection of mice against *Francisella tularensis* infection by means of passive transfer of immunity.

Materials and methods: Mice C3H/Cbi/BOM were used for transfer experiments. The spleen cells for the transfer of immunity (5×10^7 spleen cells i.v. per recipient mice) were prepared from donor mice (immunized by *Francisella tularensis* strain 15L) on day 21 after immunization. Recipient mice were irradiated by the dose of 4 Gy and reconstituted by the suspension of donor spleen cells third day after irradiation. Two hours after reconstitution the mice were infected s.c. by F. *tularensis* strain 15L in the dose 1.1×10^2 live tularemia microbes per mouse. In parallel experiment the control and immune sera were used for reconstitution instead of spleen cells. In some experiments the spleen cell suspension was treated by anti Thy 1.2 polyclonal antibody and complement or by normal murine serum plus complement. For the disruption of cells we used the sonication of donor spleen cell suspension.

Results and discussion: The 4 Gy irradiated and reconstituted mice did not survive the s.c. tularemia infection caused by strain 15L. The reconstitution of mice with spleen cells or thymus cells isolated from non-infected donors had negligible effect on their survival of infection. On the other hand the reconstitution of irradiated mice with spleen cells from immunized mice conferred "full" protection (100% of survival) of mice against s.c. F. *tularensis* infection in spite of the treatment of donor cell suspension by anti-Thy 1.2 antibody. The reconstitution with sonicated cell suspension conferred only 20 % of survival.

The transfer of immune sera to irradiated mice conferred the same degree of protection as the transfer of cells from immunized mice. "Full" protection was reached using sera obtained from immunized mice from day 7 to day 56 after immunization and by sera dilluted under the agglutinating titre. Opsonization *of F. tularensis* 15L microbes by antitularemic antisera reduced the lethal effect of infection of 30%. The challenge of irradiated, serum reconstituted mice (that survived the primary F. *tularensis* 15L infection) with *Francisella tularensis* strain SCHU (22 days after primary infection) resulted in survival of all mice infected.

Conclusions: The experiments presented herein suggested the capacity of B cells and antibodies to confer some degree of protection to irradiated hosts. The passive transfer of immunity by antibodies did not interfere with the induction of adaptive immune responses in irradiated recipients *(Macela* A., *Kopecký* J., *Propper* P., *Erichleb* M., *Vávrová* J., *Petýrek* P., *Nouza K, Šůla* K., *Neuvirt* J., *Šulc* K., *Nečas* E.: *Research Report* No.107-214-11, *Hradec* Králové,1995).

RR5 ACTIVITY OF MACROPHAGES, NK CELLS, AND LYMPHOCYTES IN THE COURSE OF *FRANCISELLA TULARENSIS* 15L INFECTION IN MICE (1985)

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Introduction: The background of induction of protective, long lasting immunity against *Francisella tularensis* microbes remains to be elucidated. The concept of participation of early innate resistance mechanisms on the resolution of primary infection and expression of cell-mediated immunity and long lasting memmory has been accepted. Among the cells that create the cellular background of immunoregulatory mechanisms operating in the course of F. *tularensis* infection, macrophages, B cell, NK cells, and several subpopulations of α/β and γ/δ T cells take part.

We have studied the metabolic and functional activities of macrophages, NK cells, and lymphocytes during late seventies and early eighties. The overall aims of the study was to map the cellular background and the sequence of events during early stages of F. *tularensis* 15L infection.

Material and Methods: Guinea pigs, outbread mice, and several inbread strains of mice were used for the study of interaction of *Francisella tularensis* strain 15L and macroorganisms. We used the electrontransfer metabolic pathways as criterion of metabolic activity of macrophages. The prolipherative activity of lymphocyte populations was tested by the blast-transformation. The ^{51}Cr release assay was used for testing of functional activity of macrophages and lymphocyte populations. Macrophage activating factors in supernatants of spleen cell cultures were studied using stepwise chromatographic separation technique.

Results and discussion: We were able to distinguish lymphocytes, "small mononuclears", monocytes and macrophages in the blood and peritoneal lavage of infected guinea pigs. The kinetics of "small mononuclears" and monocytes in the blood of infected guinea pigs was parallel up to fifth day after i.p and s.c. infection with F. *tularensis* 15L. The lymphocytes had different kinetic profile. On the basis of the kinetics of cell populations (redistribution /homing?/) we have stated that the subcutaneous infection stimulated the "lymphatic" system intensively than intraperitoneal infection *(Libich* J., *Macela* A., *Votruba* D.: *Research Report* LE-RM 12-01, Prague,1978).

The cytotoxic activities of adherent peritoneal cells and spleen cells (NK-cell) have been detected in the course of primary F. *tularensis* 15L infection of mice. The functional activity of adherent peritoneal cells (the peak 7th day after infection) was preceded by increased metabolic activity (5th day after infection). Moreover, the increased level of superoxide dismutase (SOD) was present in peritoneal cells the third day after infection. The supernatants from spleen cells prepared from infected mice were able to stimulate macrophages for metabolic and functional activities. We were able to detect some heterogeneity among macrophage activating factors in supernatants from spleen cell cultures prepared from infected mice. Some part of activity in active fractions was eliminated by monoclonal antibodies directed against IL-2, IFN-gamma, and TNF-alpha. Remaining parts of activity have the Mr 72 kDa, 52 and 15.5 kDa. We have observed the suppression of lymphoproliferative response in the course of F. *tularensis* 15L infection. By comparing this result with results obtained from experiments with another system of antigenic stimulation we suggested that the suppression of lymphoproliferative response is general phenomenon characteristic for the onset of induction of adaptive phase of immune response. The suppression of blasttransformation was possi-ble to transfer by supernatants prepared from spleen cells of infected mice to in *vitro* cultures prepared from control, noninfected mice. Separation of supernatants on Sephadex G-100 revealed the suppressive activity in fractions of Mr 28, 61 and 150 kDa. The insufficiency in production of IL-2 in this stage of infection was not primary source of suppression.

Conclusions: In our experimental systems we were able to detect the changes in the "cellular background of immune reactions very early after infection (hours), increased level od SOD (3rd day), metabolic (5th) and functional (from 7th to l4th day) activities of macrophages, activity of NK cells in spleens of infected mice (5th - l4th day), suppression of lymphoproliferation (5th), expression of DTH reaction (7th - l8th day), increased lymphoproliferative response to F. *tularensis* antigen (from day 21 postinfection), and production of antibodies from day 5 after infection. Using correlation test, from all tested reactions, only production of antibodies correlated well (k = 0,94) with the protection of immunized mice (primary infection with strain 15L) against virulent strain of *Francisella tularensis*.

RR6 APPLICATION OF PRINCIPAL COMPONENT ANALYSIS TO THE STUDY OF F. *TULARENSIS* INFECTION IN MICE ALL THE LEVEL OF MACROPHAGE PROTEINS.

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Introduction: Although many studies have been conducted into the host's immune response to *Francisella* tularensis infection, relatively little at-

tention has been focused on characterization of the alterations in host cellular protein patterns that are induced by tularemic infection and can remarkably influence ultimate result of the interaction of host and intracellular pathogen. We attempted to study the impact of tularemic infection to the host analysing cellular protein composition by two-dimensional gel electrophoresis (2DE) in the course of infection in mice.

Material and Methods: Female inbred C3H/CBi mice challenged s.c. with a live F. *tularensis* vaccine strain 15L or sterile physiological solution were used. The radiolabelled spleen cell samples prepared in various time intervals until day 10 after the infection were solubilized and separated by 2DE. Quantitative protein data obtained by computer assisted evaluation (Kepler software) were applied to Principal Component Analysis (PCA) computed by means of Statgraphic software (4.2.).

Results: From the number of polypeptides (405 - 929) separated on each gel, 62 - 73% were successfully matched. The first three components contained 36, 21, and 16% of variance, respectively, thus covering 72% of total variance comprised in our experimental set. The spatial distribution of gels in three-dimensional PCA space satisfactorily separated the untreated control, infections of days 3 and 7, while infection of day 10 were closely grouped with contol treated with a physiological solution. The proteins that may mostly contribute to the differences among individual samples analysed were selected and characterized by matching with Proteinpaedia (lymphocyte database and gene catalogue, BII, Switzerland).

Conclusion: The analysis allowed us to estimate the relatedness of cell specimens in regard to their protein patterns. The objective classification of cellular protein alterations based on PCA indicates that the most remarkable changes occur until day 7 after infection and they seem to be a direct consequence of alterations in gene expression induced by tularemic infection.