Evaluation of Immunoglobulin Production during Tularaemia Infection in BALB/c Mouse Model

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Abstract

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The chromatographic technique was used for assay of time-dependent content of antibodies in mice BALB/c infected by tularaemia. The assay was consequently performed on two types of chromatographic sorbents. The first was commercial CBind^{TM}L specific for serum immunoglobulins IgM, IgG and IgA. The second was originally prepared sorbent including protein G covalently bound on Enzacryl$_{R}$AH particles. This sorbent has specificity for immunoglobulin IgG and its subclasses only. Finally, mass concentration of immunoglobulins in serum was determined. Two curves expressing time behavior of immunoglobulin content (immunoglobulin mass concentration vs. days after immunization) were used for finding the proper mathematical function. The function found was sigmoid; subsequently, appropriate constants needed for function solution were evaluated.

Francisella tularensis, immunoglobulin, chromatography, protein L, protein G

Francisella tularensis is a small, non-motile, gram-negative bacterium living in aerobic conditions. *F. tularensis* is a well-known pathogen causing a zoonotic disease tularaemia, predominantly occurring on the northern hemisphere. Naturally occurring tularaemia disease is spread in populations of mammals such as rodents, rabbits, and hares. Typical vectors are small arthropods such as ticks (Dennis et al. 2001). Natural foci of tularaemia in the Czech Republic were described (Pikula et al. 2004). Another study presented that 2.1 - 2.8% of *Dermacentor reticulatus* in the Czech Republic and Austria are natural reservoirs of *F. tularensis* (Hubálek et al. 1998). Antibody prevalence (17%) against *F. tularensis* was found in wild boars hunted in the South Moravia region during 1993 - 1994 and compared with other years (Hubálek et al. 2002). Tularaemia can be manifested in different forms; most frequently in ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal and septic forms (Pullen and Stuart 1945).

Innate and adaptive immune response to *F. tularensis* has been extensively reviewed (Elkins et al. 2003). The progress of tularaemia infection was monitored in several organs of mice BALB/c: in the spleen, lungs and liver, and the presence of *F. tularensis* propagated viable cells in tissues of the mentioned organs was observed already a few hours after intraperitoneal or intravenous LD50 dose (Fortier et al. 1991). A generation of specific protective immunity in C3H/HeN mouse model was presented by Elkins et al. (1992). B-cell immunity response was studied in mice BALB/c model and important protection activity of this response was proved (Culkin et al. 1997). B-cell and IFN-gamma dependent response was described also for immunization by *F. tularensis* LVS outer membrane lipopolysaccharides (Dreisbach et al. 2000). Mice BALB/c exposed intranasally to *F. tularensis* LVS dose produced IFN-gamma on a large scale within 72 h; subsequently, NK cell lines responsible for IFN-gamma were characterized (Lopez et al. 2004). Another work described elevated production of IgM within two days after immunization (Cole et al. 2006). The protection of mice BALB/c after immunization of *F. tularensis* LVS was

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described elsewhere (Green et al. 2005). Cell-mediated immunity to *F. tularensis* can persist for a long time, even several years (Ericsson et al. 1994).

The monitoring of antibody production in BALB/c mouse model after exposure to *F. tularensis* LVS was the aim of this work. Analysis of antibodies was performed on one of the classical chromatographic assays: solid phase extraction (SPE) adopted for this purpose. In the previous work, the possibility of immune-response detection by piezoelectric based biosensor was extensively investigated (Pohanka et al. 2007); however, antibody quantification was not the object of this work. The development of a method for simple and reliable evaluation of antibodies count in tularaemia-affected organism remains a challenge. In the present study, quantification of immunoglobulin content is described during the infection progress, along with the search for convenient mathematical model.

Materials and Methods

Microorganism

F. tularensis LVS (ATCC 29684) was cultured on McLeod agar in a thermostat adjusted to 37.0 °C. Bovine haemoglobin and Iso VitaleXTM (Becton-Dickinson, San Jose, CA, USA) were used as supplement. Cells were harvested after one-day cultivation and suspended in physiological solution and washed two times by gentle centrifugation (2,000 × *g*) for 5 min and re-suspended in the same physiological solution. Cell concentration was adjusted to 105 CFU/ml. Concentration was measured by Cell Density Meter CO8000 (WPA, Cambridge, UK).

Mice inoculation and preparation of serum

The specific pathogen-free female mice BALB/c aged 3 months (at the beginning of the test) were obtained from BioTest (Konárovice, Czech Rep.). A total of 30 mice were divided into ten groups: nine groups were inoculated subcutaneously with 0.1 ml of 105 *F. tularensis* CFU/ml (0.1 LD50) and blood was collected at the interval of 1, 2, 4, 5, 6, 7, 10, 14 and 21 days after immunization; blood from one group was collected for normal mouse serum (NMS) preparation purposes immediately without immunization. For time expression purposes in figures, NMS is expressed as 0 days after immunization. Mice were bled under anaesthesia from cervical artery. The treatments with laboratory animals were supervised by the ethics committee.

Collected blood was incubated at 37.0 °C for 30 min. Blood clot was carefully separated and crude serum was incubated at 4 °C for another 30 min. Finally, the serum was centrifuged two times at 3,000 × *g* for 3 min.

Sorbent preparation

Two sorbents were used for the purposes of this work. Commercial CBind™L (C-L) was obtained form Fluka (Buchs, Switzerland). The application of this sorbent is based on the specificity of protein L covalently bound on cellulose particles. Protein L is able to largely interact with IgG, IgM and IgA (Akerström and Björck 1989). Second sorbent was chosen containing protein G (Sigma, St. Louis, MO, USA). Protein G binds only to IgG (all IgG subclasses) of a large group of animals - there is no cross-reactivity with other classes of antibodies (Björck and Kronvall 1984). Protein G (5 mg/ml, incubation for 4 h) was immobilized on Enzacryl^{R}AH (Sigma) according to manufacturer's instructions and after previous activation (2 h) by 1% glutaraldehyde. Potentially remaining free amino hexyl spacers were eliminated by bovine serum albumin solution (5 mg/ml for 2 h). Created Schiff bonds were reduced by $NaBH_4$ (0.5 mg/ml; 5 minute). Prepared conjugate Enzacryl^{R}AH - protein G (E-G) was washed and kept in phosphate buffered saline (PBS).

Chromatography setup

The procedure for C-L as well as E-G employing is the same. The column for SPE was filled by 1 ml of wet sorbent. All solutions were applied directly into the column. Solution flow was forced only with gravitation. The column was washed with ten column volumes of PBS, then 0.1 ml of × 10 diluted serum sample was added. After column cleaning with PBS (10 volumes of the column), captured immunoglobulins were eluted by 100 mM glycine buffer pH 2.2 (1 volume). After elution, the column was cleaned with 20 mM Tris/HCl pH 7.5 containing 6 M guanidine hydrochloride (5 volumes). Finally, 20 volumes of PBS equilibrated the column. Immediately after elution, total protein of the eluted sample was measured by the total protein kit TP0100 (Sigma). Data were processed using software Origin 6.1 (Northampton, MA, USA); non-linear fitting was used for mathematical operations purposes.

Results

Determination of column binding capacity

The column capacity was tested on a serum sample obtained 21 days after immunization (0.1 ml; × 10 diluted). This serum was repeatedly analyzed by each column. The aliquot of eluate was dissolved in × 9 the amount of PBS and measured again. The losses in second measuring cycle were under 4% (3.6 ± 0.2%). The application of different amount of sera served for estimation of column binding capacity. C-L column filled with one ml of sorbent

has proven capacity of nearly 12 mg; E-G column with the same sorbent volume had little less binding capacity: approximately 9 mg. The samples used for subsequent analysis were diluted ten times and applied in the amount of 0.1 ml, which is seventy and sixty times under maximal column binding capacity. Due to this dilution losses were minimal.

Chromatography on CBind™L (C-L) column

The mice sera were investigated for IgM, IgG and IgA presence. All ten intervals (NMS and serum collected 1 - 21 days after immunization) were consequently analyzed by the column with C-L and total protein kit. The expression of the obtained results is presented in Fig. 1 as the plotting of immunoglobulin content (mg/ml) vs. time after immunization (days). The obtained content responds to the total amount of IgG, IgM and IgA. All intervals were processed three times for statistical purposes.

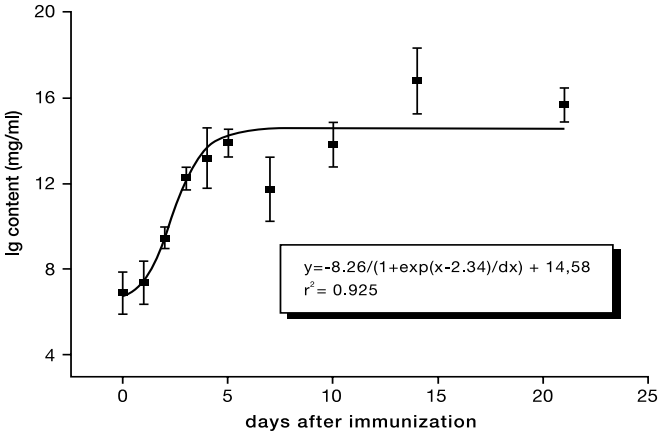

Fig. 1. Plot expressing immunoglobulins IgG, IgM and IgA content (mg/ml) in mice BALB/c model infected by tularaemia. The axis X expresses time after immunization (days), where day 0 means normal mouse serum. Chromatography based on CbindTML was used for immunoglobulin evaluation. The standard deviation (error bars) is expressed for N = 3. The curve fitting was realized in Origin 6.1. Proper mathematic function is inserted into the figure.

Non-linear fitting (Origin) was used for mathematical equation expression. Mathematical models were tested and the model with the highest correlation coefficient (r^2_{xy}) was chosen as optimal. Finally, the sigmoidal curve appeared to be the best ($r^2_{xy} = 0.937$). The generalized mathematical equation can be expressed as follows:

$$y = \frac{(A_1 - A_2)}{(1 + e^{(x-x_0)/dx})} + A_2 \qquad \text{(eq. 1)}$$

where A_1 is the bottom and A_2 is upper limit of y value. (A_1 - A_2) is the range of function on the y axis. The point x_0 responds to the point (A_1 + A_2)/2. The symbol values were computed. The constant A_1 was evaluated as 6.32 ± 1.31 mg/ml, the constant A_2 as 14.58 ± 0.47 mg/ml. The x_0 was equal to 2.34 ± 0.33 days and dx to 0.78 ± 0.29 days.

Chromatography on Enzacryl®AH - protein G (E-G) column

Similarly to C-L column, E-G column was employed for all nine intervals of sera from immunized mice and also NMS. The concentrations of only IgG in serum samples were thus obtained. The best mathematical expression of obtained concentration vs. time

dependence was again sigmoid (Fig. 2), mathematically responding to equation 1. The correlation coefficient for sigmoidal function was 0.954. The values of constants were as follows: A_1 was 5.93 ± 1.44 mg/ml, A_2: 13.51 ± 0.55 mg/ml, x_0: 4.84 ± 1.02 days and dx: 2.10 ± 0.94 days.

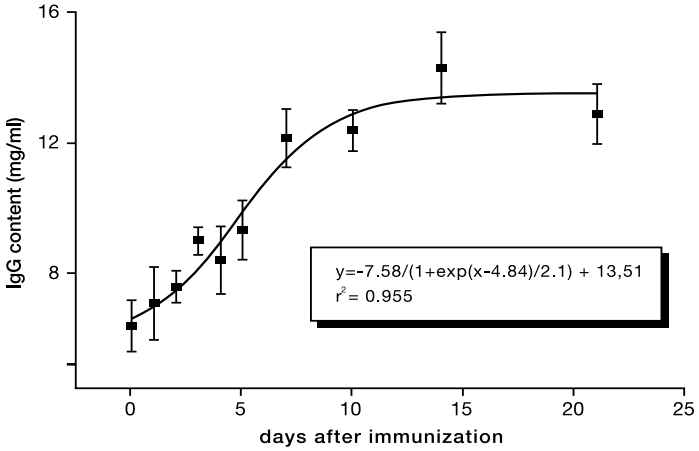

Fig. 2. The expression of IgG concentration (mg/ml) monitored for 21 days after immunization in mice BALB/c infected by tularaemia. Chromatography based on Enzacryl^{R}AH-protein G was used for IgG assay. The interval day 0 means normal mouse serum. The standard deviation (error bars) is expressed for N = 3. The curve fitting was realized in Origin 6.1. Proper mathematic function is inserted into the figure.

Discussion

The obtained results described well the antibodies rising during tularaemia infection. The selected model supposes the production of antibodies in the first 21 days after immunization and the main focus of the work was appointed at the phase where B-cells are activated according to the infection progress and the content of antibodies is elevated. Antibody degradation following within several weeks or months was not the object of this study, and the mathematical model does not describe this phase.

Let us follow the importance of values from equation 1. The limit of antibody content is expressed by constants A_1 and A_2. Antibody content before immunization (limit at zero time) is expressed by A_1; on the other side the value A_2 represents the maximal (according to the mathematical model) content of antibodies. The experimental data obtained by NMS measuring exert good correlation to A_1 limit. The amount of IgG, IgM and IgA (C-L chromatography) for NMS was measured as 6.90 ± 0.96 mg/ml; the responding value A_1 was 6.32 ± 1.31 mg/ml. Similarly, the amount of only IgG (E-G) for NMS was found to be 6.40 ± 0.78 mg/ml, which corresponds to the computed 5.93 ± 1.45 mg/ml. These obtained values overlap within their standard deviations. The values A_2 do not correspond with only one experimental value as A_1 that correspond to NMS. The value of A_2 does not consider antibody degradation, so the informational importance is only for the mentioned time lap. Difference in the amount A_1 and A_2 for C-L, respective E-G is equal to 0.39 mg/ml, respective 1.07 mg/ml. This amount corresponds to the amount of IgM and IgA in serum before or after immunization.

More general conclusion can be made from x_0 values. The value of dx has only practical importance for equation 1 solution and will not be further mentioned. However, the value x_0 is more applicable. In equation 1, x_0 has the meaning of a point at x-axis, where value

y is exactly in half between A_1 and A_2. Practically, it is a time point where the immunity response is in full production of antibodies and antibody content is in the half of maximal addition. The content of IgG, IgM and IgA commonly as mass sum reached 2.34 ± 0.33 days, only IgG reached to this point nearly five (4.94 ± 1.04) days after immunization.

The selected model described by equation 1 for sigmoid curve seems to be reliable for the infected BALB/c antibody content description. Time response included in Fig. 1 is in correlation with the symptoms that were observed on BALB/c mice after immunization. The general shape of curves presented in Figs 1 and 2 is in accord with assumptions presented in papers concerning humoral immunity, such as published by Koskela and Salminem (1985).

Vyhodnocení tvorby protilátek v modelu myší BALB/c infikovaných tularémií

Chromatografická technika byla použita k analýze časového průběhu hladiny protilátek u specifických patogenů prostých myší BALB/c infikovaných patogenním mikroorganismem *Francisella tularensis*. Pro potřebu analýzy byly použity dva typy chromatografických sorbentů. Prvním byl komerční CBindTML, vykazující specifitu k sérovým imunoglobulinům IgM, IgG a IgA. Druhým byl pro tyto účely připravený sorbent sestávající z proteinu G kovalentně navázaného na částice EnzacrylRAH. Tento sorbent byl specifický pouze k imunoglobulinům IgG a jeho podtřídám. Následně byla určena výsledná hmotnostní koncentrace imunoglobulinů v séru. Křivky obsahující časový průběh hladiny imunoglobulinů byly použity pro vyhledání náležité matematické funkce. Tato funkce byla shledána jako sigmoidní a následně byly vyhodnoceny konstanty důležité pro řešení funkce.

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