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Research Article

Cell Envelopes of *Francisella tularensis*: Immunogenic Activity and Toxicity

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Abstract

At present, development of effective vaccines of new generation is an actual problem, in particular concerning the tularemia causative agent. It determines the need to search antigen determinants with high immunogenic activity. Some authors demonstrate that outer membrane proteins of *Francisella tularensis* possess immunological activity. This fact gave occasion to isolation and comprehensive study of *Francisella tularensis* cellular envelopes as a perspective component in vaccine engineering.

Toxicity and immunogenic activity of Cell Envelopes (CE) of *Francisella tularensis* different subspecies is complex studied. It is shown that these preparations in doses 6.3; 19.0; 57.0 and 100 μg fail to possess toxicity. The immunizing dose (95 μg) of CE *Francisella tularensis* subsp. *holarctica* 306, *Francisella tularensis* subsp. *mediasiatica* A-61 and *Francisella tularensis* subsp. *tularensis* B-399 A-Cole protecting white mice against experimental tularemia infection was determined.

It is experimentally demonstrated that 83 % animals survive only after using CE *Francisella tularensis* subsp. *mediasiatica* A-61. It is shown that *F. tularensis* CE preparations influence on activation of T and B lympho-cytes of white mice blood cells.

New data concerning the possibility of *F. tularensis* antigen preparation application for increase of experimental animal resistance to *F. tularensis* are obtained. On the basis of the findings there is need for the further detailed investigation of immunogenic properties of CE *F. tularensis* subsp. *holarctica* 306, *F. tularensis* subsp. *mediasiatica* A-61 and *F. tularensis* subsp. *tularensis* B-399 A-Cole as perspective components in development of tularemia vaccines.

Keywords: Cell envelope; Flow cytometry; *Francisella tularensis*; Immunity

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Introduction

At present vaccinal prevention as a priority system of measures aimed at the warning, restriction of distribution and elimination of especially dangerous infectious diseases is of special importance. Live attenuated vaccines developed and introduced in practice still in the XX century including tularemia vaccine are used as vaccinal preparations for specific prophylaxis of these illnesses. In this connection development of new generation vaccines possessing high immunological and epidemiological efficiency is an actual direction of researches.

The basic antigen determinants are located on surface of *Francisella tularensis* bacterial cells. So, one of *F. tularensis* immunodominant antigens is Lipopolysaccharide (LPS). However, there are contradicting data in the literature concerning properties of *F. tularensis* external membrane components that can be explained by various methods of its isolation and investigation conditions. There are data that *F. tularensis* LPS does not stimulate immunocompetent cells [1]. Nevertheless, other researchers established activation of mice immunocompetent cells by *F. tularensis* LPS in case of its dose increase in comparison with standard preparations [2,3]. A number of authors demonstrate that *F. tularensis* outer membrane proteins possess immunological activity [4,5], more than 30 immunoreactive tularemia agent antigens are included into its composition. It is known that *F. tularensis* outer membrane proteins influence on specificity of macroorganism immune response, possess unique polyepitope antigenic structure and are considered as perspective components in engineering of subunit chemical vaccines.

The aforesaid has formed the basis for isolation and comprehensive studying of *F. tularensis* cell envelopes as a perspective component in vaccine developments [6,7].

The aim of this investigation included the detection of toxicity of the resulted CE *F. tularensis* different subspecies and its immunizing dose with subsequent estimation of the functional activity of experimental animal blood cells under the influence of these preparations *in vivo* and *in vitro*.

Materials and Methods

Cell envelopes of *F. tularensis*: Cell Envelopes (CE) prepared by lysis of live cells from 6 *F. tularensis* strains: *F. tularensis* subsp. *novicida* Utah 112 [6], *F. tularensis* subsp. *mediasiatica* A-61, *F. tularensis* subsp. *tularensis* B-399 A-Cole, *F. tularensis* subsp. *holarctica* 21/400, *F. tularensis* subsp. *holarctica* 306, *F. tularensis* subsp. *holarctica* 15 NIIEG from the collection of Irkutsk Antiplague Research Institute were used in this work.

Experimental animals: The tests were performed using adult certificated outbred white mice (from Scientific Production Association "Vektor", Novosibirsk city) both sexes, weighing 22-24 g. Manipulations with experimental animals carried out in concordance with the international principles of the Helsinki declaration concerning to the humane relation to animals.

Immunizing Dose (ID) and protective activity: Immunizing Dose (ID) и protective activity of CE was detected using 114 white mice.

Animals were divided into six experimental groups in 18 white mice in each group and one control in 6 mice. *F. tularensis* CE preparations were injected subcutaneously into the right leg in volume 0.5 ml in three doses: 3.8; 19 and 95 μg (at recalculation to protein). Control animals were injected the same volume of NaCl isotonic solution, pH 7.2. At 28 days after immunization white mice were injected subcutaneously a bacterial suspension of high virulent *F. tularensis* subsp. *holarctica* 306 (Dcl 1) strain in dose of 100 CFU per an animal.

Toxicity of CE *F. tularensis*: Toxicity of CE *F. tularensis* different subspecies was determined in 125 white mice as experimental models. Animals were divided into 24 experimental and one control groups (5 mice in each group). White mice were immunized once subcutaneously with CE preparations of *F. tularensis* different subspecies in the right leg in volume of 0.5 ml in a dose 6.3; 19.0; 57.0 and 100 μg (at recalculation to protein). Control animals were injected the same volume of NaCl isotonic solution, pH 7.2.

Flow cytometry: Functional activity of blood cells was examined by a flow cytometry method *in vivo* and *in vitro*. Total 120 white mice were used *in vitro* experiments. Leukocytes isolated from heparinized blood using 3 % gelatin were tested. Cells in concentration of 10^6 (50 μl) were incubated during 60 min with CE *F. tularensis* preparations in two doses (19 and 95 μg to protein). Immunogenic activity of CE *F. tularensis* was examined in 60 white mice *in vivo*. In this case the animals were immunized with CE *F. tularensis* preparations in dose 95 μg/0.2 ml of buffered physiological solution per an animal. Blood sampling was performed in 3 days after injection of the experimental preparations. The phenotype of white mice blood cells was defined using monoclonal antibodies by Becton Dickinson Company (USA) in the following panel: CD45-APC/CD3-FITC/CD4-Alexa-700/CD8-APC-Cy7/CD25-PE-Cy7/Annexin-PE/7-AAD. Stained samples were analyzed on flow cytofluorometer BD FACSCanto ™ II. Total activated T-lymphocytes ($CD3^+CD25^+$), activated T-helpers ($CD3^+CD4^+CD25^+$) and activated B-lymphocytes ($CD3^-CD19^+CD25^+$) were detected. Also viability of the cell populations by percentage of live cells ($AnV^-7\text{-}AAD^-$), necrotic cells ($AnV^-7\text{-}AAD^+$), cells at the stage of early ($AnV^+7\text{-}AAD^-$) and late ($AnV^+7\text{-}AAD^+$) apoptosis was estimated *in vitro*.

Statistical analysis: Statistical data were processed using parametrical t-criterion with the Bonferroni adjustment. $P < 0.05$ was considered significant.

Results

It was shown that cell envelope preparations of *F. tularensis* different subspecies in doses 6.3; 19.0; 57.0 and 100 μg were non-toxic for experimental animals (LD_{50} is not determined). Dissection at the place of the preparation injections revealed no alterations in kidneys, adrenal glands, lungs, heart and other organs. At the same time, alterations in spleen and lymph nodes were observed in case of *F. tularensis* subsp. *holarctica* 15 NIIEG, CE *F. tularensis* subsp. *novicida* Utah 112, *F. tularensis* subsp. *tularensis* B-399 A-Cole and *F. tularensis* subsp. *mediasiatica* A-61 injections indicating immune reorganization of organism.

Experiments for ID (Table 1) detection of the tested preparations showed that CE *F. tularensis* subsp. *holarctica* 306, *F. tularensis* subsp. *mediasiatica* A-61 and *F. tularensis* subsp. *tularensis* B-399 A-Cole in dose of 95 μg protected experimental animals in the infectious process caused by high virulent *F. tularensis* subsp. *holarctica* 306 strain. Thus only in case of CE *F. tularensis* subsp. *mediasiatica* A-61 application the survival rate of the animals was 83 %, CE *F. tularensis* subsp. *holarctica* 306 - 33 %, CE *F. tularensis* subsp. *tularensis* B-399 A-Cole - 17 %.

CE *F. tularensis*	ID, mkg	Number of animals			ID_{50} (mkg)
		Tested	Died	Survived	
F. tularensis	95	6	6	0	95<
subsp. holarctica	19	6	6	0	
15 NIIEG	3.8	6	6	0	
F. tularensis	95	6	6	0	95<
subsp. holarctica	19	6	6	0	
21/400	3.8	6	6	0	
F. tularensis	95	6	4	2	124.94
subsp. holarctica	19	6	6	0	
306	3.8	6	6	0	
F. tularensis	95	6	6	0	164.18
subsp. Novicida	19	6	6	0	
Utah 112	3.8	6	5	1	
F. tularensis	95	6	1	5	43.17
subsp. Mediaasiatica	19	6	5	1	
A-61	3.8	6	6	0	
F. tularensis	95	6	5	1	164.18
subsp. Tularensis	19	6	6	0	
B-399 A-Cole	3.8	6	6	0	
контроль		6	5	0	

Table 1: Immunizing dose and protective activity of cell envelopes after *F. tularensis* subsp. *holarctica* 306 subcutaneous injection to experimental animals.

The results demonstrated that the experimental *F. tularensis* preparations in doses of 19 and 95 μg/10^6 leukocytes *in vitro* failed to affect statistically on the expression level of early activation marker (CD25) of T- and B-lymphocytes. Preparations of CE *F. tularensis* different subspecies in these doses did not influence on necrosis and apoptosis processes in experimental animal blood cells.

Experiments *in vivo* showed that statistically significant ($P < 0.05$) increase of activated B-lymphocytes in comparison with the controls (2.6 ± 0.4 %) was registered in 3 days after white mice immunization with CE *F. tularensis* subsp. *mediasiatica* A-61 (4.1 ± 0.6 %) and *F. tularensis* subsp. *tularensis* B-399 A-Cole (3.4 ± 0.8 %). At the same time CE *F. tularensis* subsp. *holarctica* 15 NIIEG and *F. tularensis* subsp. *holarctica* 306 promoted the percentage increase of T- lymphocytes in 1.2 times (Table 2).

Discussion

It is known that macrophages are important in development of the infectious process caused by *F. tularensis* being both a primary target and also a site of bacteria localization and reproduction. Activation of intracellular signal pathways of immunocompetent cells causing the secretion of cytokines and chemokines is mediated by interaction of microbic components with pattern-discriminating macrophage receptors. So, Bacterial Lipopeptide (BLP), lipoproteins, Lipopolysaccharide (LPS) and also many other proteins (FopA, FopC, TUL4, FslE, FmvB and others) are located on surface of bacterial cells, in particular

CE *F. tularensis*	Index, %		
	$CD3^+CD25^+$	$CD3^+CD4^+CD25^+$	$CD3^-CD19^+CD25^+$
F. tularensis subsp. holarctica 15 NIIEG	11.1 ± 0.9*	9.7 ± 0.6	2.9 ± 0.3
F. tularensis subsp. holarctica 306	12.0 ± 0.8*	9.9 ± 0.5	2.4 ± 0.3
F. tularensis subsp. novicida Utah 112	10.1 ± 0.8	7.0 ± 0.6	2.6 ± 0.4
F. tularensis subsp. mediaasiatica A-61	8.3 ± 1.0	7.1 ± 0.7	4.1 ± 0.6*
F. tularensis subsp. tularensis B-399 A-Cole	8.0 ± 0.9	7.4 ± 0.7	3.4 ± 0.8*
Conrtol	9.8 ± 0.7	8.2 ± 0.8	2.6 ± 0.4

Table 2: Functional activity of blood lymphocytes of white mice immunized by CE *F. tularensis*.

Note: * – P < 0.05

F. tularensis. Due to outer membrane proteins possess higher immunogenic activity in comparison with LPS [4], studying of CE *F. tularensis* is a fundamental basis for interpretation of intracellular survival mechanisms, interaction of bacteria with macroorganism cells and a perspective direction in search of components for development of tularemia vaccine.

The conducted examinations demonstrated that CE from *F. tularensis* subsp. *novicida* Utah 112, *F. tularensis* subsp. *mediasiatica* A-61, *F. tularensis* subsp. *tularensis* B-399 A-Cole, *F. tularensis* subsp. *holarctica* 21/400, *F. tularensis* subsp. *holarctica* 306, *F. tularensis* subsp. *holarctica* 15 NIIEG strains in doses 6.3; 19.0; 57.0 and 100 μg were non-toxic for experimental animals. This fact was confirmed by the results of pathomorphological analysis of white mice organs immunized with these preparations and also by the indicators of blood cell viability at interaction with CE *F. tularensis in vitro*.

The immunizing dose (95 μg) of *F. tularensis* subsp. *holarctica* 306, *F. tularensis* subsp. *mediasiatica* A-61 and *F. tularensis* subsp. *tularensis* B-399 A-Cole protecting white mice from experimental tularemia infection was established.

There are literature data that the preparations containing *F. tularensis* external membrane proteins possess immunological activity in 19 μg dose [4,5]. In this connection CE *F. tularensis* in two doses: 19 and 95 μg was used in our experiments to estimate functional activity of blood cells of the laboratory animals by flow cytometry method. It was experimentally demonstrated that CE *F. tularensis* preparations rendered multidirectional influence on activation of T- and B-lymphocytes of white mice blood cells. So, CE *F. tularensis* subsp. *mediasiatica* A-61 and *F. tularensis* subsp. *tularensis* B-399 A-Cole preparations possessing immunogenic activity (the survival rate was 83 % and 17 % of animals, respectively) resulted in increase of $CD3^-CD19^+CD25^+$-lymphocyte percentage, and *F. tularensis* subsp. *holarctica* 306 CE (33 %) - $CD3^+CD25^+$- lymphocyte.

The distinctions in protective activity of the preparations and their ability to increase functional activity of white mice lymphocytes determined during the examination can be possibly caused by nucleotide polymorphism of the genes coding outer membrane proteins [8], and variability of LPS structure [9,10] in *F. tularensis* subspecies that undoubtedly affects on the bacteria virulence.

Formerly we determined that CE *F. tularensis* stimulated IL-1β, TNF-α, IL-2, GM-CSF and G-CSF production by immunocompetent cells of the experimental animals *in vivo* [6,7].

On the basis of the conducted investigations it is possible to conclude that CE preparations of *F. tularensis* subsp. *holarctica* 306, *F. tularensis* subsp. *mediasiatica* A-61 and *F. tularensis* subsp. *tularensis* B-399 A-Cole possess protective activity, ability to increase functional activity of blood lymphocytes and stimulate production of proinflammatory cytokines by white mice immunocompetent cells. At the same time the further complex research of immunogenic properties of these preparations as perspective components in development of tularemia vaccines is required.

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