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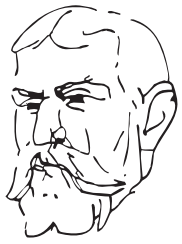

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ORIGINAL ARTICLE
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ANAPLASMOSIS: EXPERIMENTAL IMMUNODEFICIENT STATE MODEL

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ABSTRACT

Introduction: The recently described anaplasmosis infection is widespread but concerns to the insufficiently known group of diseases.

The aim of our research is the development of uniform biological model for reproducing of artificial immunodeficient state by experimental anaplasmosis.

Materials and methods: Algorithm of experimental anaplasmosis reproducing, consisted of such consecutive stages: 1) artificial forming of the immunodeficient state at nonlinear white mise (*Mus musculus* L.); 2) preparation of the tested biological material samples; 3) inoculation by prepared samples of the laboratory animals with the artificially formed immunodeficient state; 4) sampling from the dead or slaughtered (by the method of chloroformed anesthesia) experimental animals of sectional material (organs and targets tissues); 5) verification of aetiology by express detection of causative agents by the method of PCR in the selected samples of sectional material.

Results: Biological model of experimental anaplasmosis have been created suitable for realization of both diagnostic and epidemiological, epizootic, ecobiological and other researches of different origin biological material samples, including samples of solid and liquid consistency material. Formed model realised in premature death of experimental animals in 17.4 % cases; resulted in an onset of disease clinical signs without death during the term of supervision in 43.8 % cases; coursed in the absence of the expressed symptoms of infection in 31.3 % cases.

Conclusions: Developed biological model of experimental anaplasmosis consists in that as laboratory animals with the increased sensitiveness to the infection and accumulation of causative agent are used white nonlinear mice with the artificially formed immunodeficient state.

KEY WORDS: biological model, *Mus musculus* L., cyclophosphamide, anaplasmosis, *Anaplasma phagocytophilum*

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INTRODUCTION

The recently described anaplasmosis infection is enough widespread in the European countries concern to the insufficiently known group of diseases. Anaplasmosis is included in the group of transmissible infectious diseases of man, cattle and mammals, and caused by bacteria of the genus *Anaplasma* [1-4]. A decision value in diagnostics of the disease is taken the methods of etiologic verification of anaplasmosis, so as clinical course is characterized only by the syndrome of general intoxication and, as a rule, not accompanied by specific clinical displays [5-7].

Laboratory models for an artificial reproducing of anaplasmosis at the experimental animals are actively developed in different countries [8, 9]. The ultimate goals of such researches are to study pathogenesis features; identification of patterns of immunological reactions and formation of specific and nonspecific immunity; determination *in vivo* conditions of etiologic diagnostics methods effectiveness (specificity, sensitiveness, producibility); efficiency of etiotropic therapy and methods of specific immunoprophylaxis; identification of natural reservoir of infection and mechanisms of pathogen transmission.

THE AIM

A research purpose is the development of uniform biological model for reproducing of artificial immunodeficient state by experimental anaplasmosis that is characterized by simplicity of technological reconstruction, cheapness and availability for wide practical usage.

MATERIALS AND METHODS

Algorithm of experimental anaplasmosis reproducing, consisted of such consecutive stages: 1) artificial forming of the immunodeficient state at nonlinear white mise (*Mus musculus* L.); 2) preparation of the tested biological material samples; 3) inoculation by prepared samples of the laboratory animals with the artificially formed immunodeficient state; 4) sampling from the dead or slaughtered (by the method of chloroformed anesthesia) experimental animals of sectional material (organs and targets tissues); 5) verification of aetiology by express detection of causative agents by the method of PCR in the selected samples of sectional material.

The artificial immunodeficient state for white nonlinear laboratory animals was created by a single intracutaneous injection of 250 μg/kg of cyclophosphamide in the form

of medicinal preparation "Cyclophosphanum®" (publicly traded company "Kyivmedpreparation", Kyiv, Ukraine) 3-4 hours before introduction of samples of the investigated biological material to animals. The intracutaneous method of preparation introduction (unlike intraperitoneal) provided more even entering of preparation to the system of blood circulation and hemopoietic organs, reduced speed of preparation elimination from the animal organism, that provided forming of more protracted at times (to 7-10 days) immunodeficient state, sufficient for reproduction and accumulation of *Anaplasma*. Optimal fixed by us empiric dose of preparation is 250 μg/kg. The injection of this dose provides forming of the enough expressed and stable immunodeficient state, and does not result in unforeseeable death of part (about 12 % and more) of experimental animals, that was marked at introduction of 500 μg/kg and more.

The selected samples of blood brought in sterile capacities with an anticoagulant (with absent antimicrobial activity) for prevention of blood coagulation. During realization of our researches we used vacuum systems of blood sampling Venosafe™, Terumo Europe N.V. (Belgium) and test tubes VF - 052SDK (working volume of 2 ml) with the anticoagulant K2-EDTA. The samples of blood were frozen by single-phase at the temperature of -20 °C and unfrozen at a room temperature that provided destruction of blood cells and release of pathogen microcolonies. The samples of biological material of solid consistency carefully homogenized (by trituration or grinding) and diluted with sterile distilled water in 1:9 ratio (volume/volume, respectively) with further mixing for formation of even suspension. Before homogenization, the surface of ticks was disinfected by immersion of them in 70 % ethyl spirit on 10 minutes. We homogenized all body of the tick, as causative agents could be in its different tissues and organs (salivary glands, lymph, intestine etc.). For introduction to the laboratory animals, we select the supernatant (after desilting and unassisted precipitation of fair-sized particles) of the homogenized samples suspension. All manipulations at a selection and preparation for research of biological material samples were carried out in aseptic conditions, for prevention of their additional contamination by extraneous microflora.

Samples of different biological material (in a volume 0,3 ml) were injected to nonlinear white mice with the artificially formed immunodeficient state intraperitonealy, that provided a large area for the application of causative agents with high authenticity of their contact with target-cells (macrophages, leucocytes, erythrocytes etc.). The term of observation of the infected experimental animals lasted 8-10 days as, in this period the concentration of causative agents arrives at a maximal value in tissues and organs of animals [8].

For exact verification of infectious process aetiology from the dead and slaughtered animals (by a method of chloroform hyperanesthetization), applied the most accessible and technologically cheap standard variant of PCR. Verification of ethology is based on the PCR detection of basic clinically meaningful types of *Anaplasma* (*A. phagocytophilum*) in the samples of blood (selected by puncture of heart with the observance of asepsis rules) of experimental animals infected by the investigated biological material samples. Expediency of exactly selection of blood samples is grounded by simplicity of executable for this purpose manipulations, and also complete coincidence of our PCR results (positive and negative) at parallel research of blood and other tissues and organs (spleen, bone marrow, liver, lymph nodes) samples, selected from the same experimental animals. We applied for reproducing standard PCR accessible on the territory of Ukraine, relatively cheap and already geared-up for the direct use of commercial sets of reagents "IsoGene Lab. Ltd" (Moscow, Russian federation): "Universal probepreparation reagents kit" – Diatom®DNA Prep 100 (includes reagents for a selection and cleansing of DNA from the samples of biological material with the purpose of further amplification of its certain fragment); " DNA amplification reagents kit" – Gene Pak®DNA PCR test: E2136 (includes reaction mixtures, with primer system *Eph* for amplification of specific fragment of *A. phagocytophilum* genome); "Marker of DNA molecular mass M50, M100" – GenePak™ DNA Ladder M50, M100 (includes DNA fragments mixtures of different molecular mass which differs on 50 or 100 pair of nucleotides, respectively, and used for size comparative identification of formed amplicons at the reproducing of PCR with synthesized primers); "Universal inner control UVK-90" (includes the reagents sets for monitoring of possible DNA losses during its selection from the investigated samples of biological material and for determination of possible inhibition of PCR in the process of its reproducing) [10].

The procedure was done strictly in compliance with the Helsinki Declaration, European Convention for the protection of vertebrate animals (18.03.1986), European Economic Society Council Directive on the Protection of Vertebrate Animals (24.11.1986) after approval from the Regional Ethical Review Board.

Statistical analyses were performed using the chi-square test while comparing categorical variables. For all analyses, $p<0.05$ was used to indicate statistical significance.

RESULTS

Biological model of experimental anaplasmosis created by us is suitable for realization of both diagnostic and epidemiological, epizootic, ecobiological and other researches of different origin biological material samples, including samples of solid and liquid consistency material, with the absence and with the presence of contamination by an extraneous microflora.

Under supervision of animals during an experiment appeared their premature death and onset of clinical signs of the disease: decline of mobility and appetite, inertness at a tactile and sound irritation, hunched seat, lameness, flatulence, loss an about 30 % of body mass. We conduct determination and comparison of morbidity level (premature death and disease incidence) in three different groups of laboratory animals: group №1 are control intact animals;

group №2 are control animals with the artificially formed immunodeficient state; group №3 are experimental animals with the immunodeficient state infected by the samples of the investigated biological material. The results of these researches showed that unlike control animals from groups №1 and №2, the experimental animals of group №3 produced clinically expressed disease. Thus in future it was set at the usage of PCR method, that anaplasmosis, was the reason of premature death of experimental animals in 17,4 % cases; resulted in an onset of disease clinical signs without death during the term of supervision in 43,8 % cases; coursed in the absence of the expressed symptoms of infection in 31,3 % cases.

For confirmation of reproduction and accumulation of *Anaplasma* in experimental animals of group №3 comparative (taking into account the positive result of PCR) determination of corpuscular antigen amounts (cells and microcolonies) of *Anaplasma* spp. bacteria was conducted by the method of indirect immunofluorescence assay in the samples of the investigated biological material, which was inoculated to experimental animals, and in the samples of sectional material from the last – tissues and organs which can contain most of the causative agents cells (blood, spleen, bone marrow, liver, lymph nodes) potentially. The results of these researches showed the accumulation of bacteria in tissues and target organs of the infected animals, which exceeded the initial concentrations of these microorganisms in the corresponding samples of the investigated biological material more than in 10^2-10^3 times. In addition, comparative analysis of the results of *Anaplasma* detection by PCR method in the same samples of biological material without the usage and with the usage of the pathogen reproduction and accumulation stage by growing in *in vivo* conditions (offered biological model) demonstrates the substantial ($p<0,05$) increase of causative agents detection from 6,9 % to 18,4 %.

DISCUSSION

First of all researchers use at creation of experimental anaplasmosis biological models those types of animals, which are natural owners for causative agents: horses, cattle, sheep, white-tailed deer, dogs, pack rats and white-footed mice [8, 9]. The usage of the indicated animals in an experiment allows recreating the natural course of infectious process with large authenticity, that allow to explain peculiarities of course for different diseases in condition of immunodeficient state [11, 12]. However, it has substantial defects, related to their inaccessibility for wide application in practice. high cost of such animals, difficulties of their maintenance in laboratory conditions and technical complications of experiment realization on them, and also by limitations in possibility of standardizated laboratory model creation for all *Anaplasma* species, that associated with ability of every species of pathogen to multiply and accumulate only in the certain type of sensible animals.

Therefore, the last years noted tendency to the increase of researches, pointed at development technically of more suitable models of experimentally reproducing anaplasmosis, based on the usage of the most widespread laboratory animals, – mostly mice, considerably rare – rats, guinea-pigs, rabbits [13]. It should be noted that for ordinary white nonlinear laboratory mice experimental anaplasmosis, characterized by complete absence or extraordinarily poorly expressed symptoms of disease without substantial reproduction and accumulation of causative agents in their organism, rapid elimination of them even in the conditions of bacteraemia in experimental animals on the initial stage after their infection. The indicated circumstances induce researchers to apply the genetically cloned syngeneic lines of mice with an innate immunodeficit and that characterized by the high level of sensitiveness to the causative agents of different infectious diseases [14]. However, such linear animals are relatively expensive, scarce enough; need the special terms for their isolated maintenance and reproduction, which would prevent deaths of animals at the casual infecting. It substantially complicates the stable providing of laboratories and their practical deployment with linear immunosuppressive animals as a biological model. Therefore, potentially most perspective for wide practical application there can be a biological method, based on the usage of white nonlinear laboratory mice with the induced immunodeficient state. It is succeeded to form by the way of introduction to the experimental animal of compounds, which have a necessary spectrum of immunodepressive action. Today there is a large list of medicinal preparations, which are characterized by the polytypical mechanisms of immunosuppression without the display of antibacterial activity. It allows carrying out a reasonable choice for application of exactly those preparations that provide the adequate reproducing of the necessary immunodepressive state for experimental animals with absence of undesirable bactericidal or bacteriostatic action on *Anaplasma*.

Choice of preparation "Cyclophosphanum®" (publicly traded company "Kyivmedpreparation", Kyiv, Ukraine) for the artificial forming of the immunodeficient state for white nonlinear mice is reasonable due to the detailed study of its mechanisms of immunodepressive action [14] and by the absence of antibacterial activity, cheapness and availability.

CONCLUSIONS

1. Developed biological model of experimental anaplasmosis consists in that as laboratory animals with the increased sensitiveness to the infection and accumulation of causative agent are used white nonlinear mice with the artificially formed immunodeficient state.
2. Biological model, based on intracutaneous introduction to the laboratory animals of immunodepressive preparation "Cyclophosphanum®" in a dose 250 μg/kg, is characterized by commonality and simplicity of reproducing, considerably cheaper than analogues and accessible for practical deployment.
3. Inoculation to white nonlinear mice with the artificially formed immunodeficient state of biological material samples, potentially infected by *Anaplasma*, allows

promoting the level of causative agents detection (with further verification by PCR method) in 2 times, that can be used for anaplasmosis diagnostics in people and animals by detection of causative agents in the samples of biological material.

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