IMMUNOPROPHYLAXIS OF SECONDARY ALVEOLAR ECHINOCOCCOSIS BY A IMMUNOSTIMULATION RIBOTAN COMBINED WITH ANTIGEN PROTOSCOLEX CELLS OF ECHINOCOCCUS MULTILOCULARIS

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Abstract: To assess the protective efficiency of immunization against secondary alveolar echinococcosis, with an antigen from Echinococcus multilocularis protoscoleces in combination with Ribotan immunostimulation, an assay was carried out on 48 white outbred mice. The use of immunostimulation in combination with specific antigens for the immunoprophylaxis against experimental alveolar echinococcosis has a synergistic effect on the treatment.

Keywords: Immunostimulation, alveolar echinococcosis, antigen, Echinococcus multilocularis, immunoprophylaxis, experimental

1 Introduction

Alveolar hydatid disease (AHD), is a parasitic zoonosis resulting from Echinococcus multilocularis s.l., a tapeworm that belongs to the class Cestoda, family Taeniidae. AHD is found worldwide, mostly in northern latitudes in central Europe, Russia, China, Central Asia, Japan, and North America. The life cycle of these parasitic tapeworm requires two mammalian hosts. First - definitive host, such as foxes and dogs (and other canids) in which the adult or strobilar phase develops in the small intestine, and second - intermediate host, in which the alveolar stage develops in different organs in the body by tumor-like or cyst-like metacestode. The larval stage of Echinococcus multilocularis causes alveolar echinococcosis, the serious helminthozoonosis with a high mortality in patients with late treatment [Wilson, 1995]. Alveolar echinococcosis is a serious disease has a significantly high fatality rate.

The therapy of this disease represents an important problem. Scientific search for effective means of protection against this deadly disease is conducted throughout the world. E.multilocularis induces parasite-specific cellular and humoral immune response in intermediate host [5]. Cell-mediated immune response depending on interaction of macrophages and T lymphocytes is regarded as protective against E. multilocularis infection [10]. Also in the scientific literature there are sufficient data on the effectiveness of various adjuvant agents such as Freund's adjuvant, aluminum hydroxide, BCG vaccine and others. These drugs are used in vaccines against parasitic infections, including larval hydatid disease [3, 6, 8, 15]. There is a lot of references to strengthen the body's resistance to helminth infection in the application of non-specific immune stimulating agents [9; 13]. Application of immunomodulatory substances could improve host immune status during E. multilocularis infection and limit the growth of the parasite [4]. The combination of specific and non-specific stimulation means provides natural resistance and increased immunoreactivity to specific antigens E.multilocularis [2].

In our study, we focused on the study immunoprophylaxis activity of immunomodulatory Ribotan in complex excretory-secretory antigen of protoscolex cells of Echinococcus multilocularis in enhancing of the host antiparasite defence in alveolar echinococcosis. Also we evaluated the protective effect immunostimulant Ribotan in experimental secondary alveolar echinococcosis of albino outbred mice.

2 Material and methods

To assess the protective efficiency of immunization against secondary alveolar echinococcosis, with an antigen from Echinococcus multilocularis protoscolexes in combination with Ribotan immunostimulation, an assay was carried out on 48 white outbred mice, males, weighing 20–25 g. These were divided into four groups of twelve mice each. Mice were kept under a 12-h light/dark regime at room temperature (21±3°C) and 50–60% relative humidity on a commercial diet and water. The experimental protocols complied with the current Russian ethics law.

E. multilocularis metacestodes (an Asian genotype circulating in the Central region of Russia; strain provided by Department of immunodiagnostic and cellular technology, All-Russian research institute of fundamental and applied parasitology of animals and plants of name K.I. Scriabina, Moscow, Russia) was obtained from the wild foxes (Vulpes vulpes) [1] and was passaged in our laboratory by intraperitoneal injection of laboratory albino outbred rats Rattus norvegicus

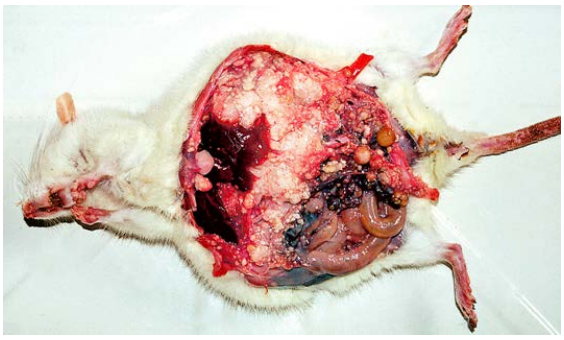
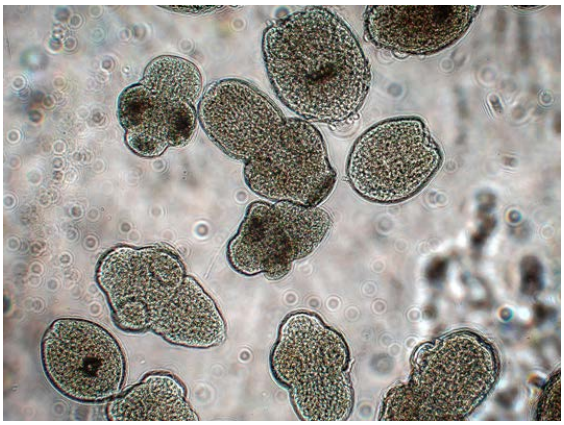


Fig. 1 Secondary alveolar echinococcosis of albino laboratory donor- rat 6 months post infection (p.i.) by intraperitoneal injection protoscolexes Echinococcus multilocularis

Figure 1 presents results of intraperitoneal injection protoscolexes E.multilocularis after 6 months post infection. We can see that the intraperitoneal infection leads to the development biggest larval stages (alveolar metacestodes) in different organs as tumor-like or cyst-like formations.

Parasite cysts were isolated 6 months post infection (p.i.) and cut into pieces in sterile 0.9% NaCl with antibiotics, 8 μg/100 ml Gentamycinum (Paneco-ltd, Russia), and passed through a nylons mesh using apertures ranging №033. Protoscolexes obtained after the last filtration were subjected to grinding and homogenization. Primary cell cultures were obtained by gentle hand-homogenization and passed through nylons cell mesh using apertures ranging from 220 to 50 μm.



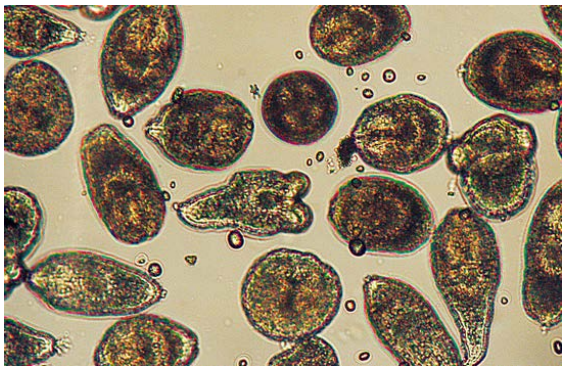


Fig. 2 Protoscolexes of Echinococcus multilocularis

The figure above shows isolated from cysts protoscolexes of Echinococcus multilocularis before their homogenization.

The antigen was isolated from the metabolism products of Echinococcus multilocularis protoscolexes cells, cultured in RPMI 1640 medium (Sigma-Aldrich, Germany) enriched with 6% fetal bovine serum. Culturing cells were performed in 6-well culture plates placed in a Heraeus CO2 incubator under conditions of high humidity, temperature of 37 °C and a CO2 level of 5% [11].

Ribotan is a complex immunostimulator consisting of a mixture of low-molecular-weight, 0.5 — 1.0 kD, polypeptides (at least 10 mcg/ml) and low-molecular-weight RNA fragments (at least 105 mcg/ml) This drug is a broad spectrum of biological activity: Ribotan stimulates natural resistance factors, leucopoiesis, migration and cooperation of T-and B-lymphocytes, as well phagocytic activity of macrophages and neutrophils. It is accelerates the formation of post-vaccination immunity, increases its intensity and duration, increases immunological efficacy of vaccines. Ribotan increases lysozyme content properdin antibody levels, induces the synthesis of interferon [14]. Designed by organization "Closed Joint-Stock Company of Research and Production firm veterinary and fur center" Vetzverotsentr ", Moscow, Russia.

The efficacy of immunoprophylaxis was evaluated by the cyst development in infected mice. E. multilocularis cysts were isolated from sacrificed mice and parasite cysts were weighed and proved pathogenicity of metacestodes by a biological test on other mice.

The mice were immunized via two subcutaneous injections, administered with a ten day interval between them. The formulations consisted of antigen protein (60 µg) and/or Ribotan (5 µl) in 0.2 ml of sterile 0.9% NaCl per injection.

Table 1. Details of the experimental evaluation of the protective properties of the complex formulation against secondary echinococcosis

The formulations in 0.2 ml of sterile 0.9%NaCl per injection	*Number of group*	*Number of animals per group*	*Units of E. multilocularis protoscolexes per mouse*
ribotan (5 µl)	group 1	12	750 ± 50
antigen protein (60 µg)	group 2	12	750 ± 50
antigen protein (60 µg) and ribotan (5 µl)	group 3	12	750 ± 50
0.2 ml of sterile 0.9% NaCl.	group 4	12	750 ± 50

The above table 1 presents the details of the evaluation of the experiment protective properties of complex drug against secondary echinococcosis. The group 1 mice were treated with the Ribotan formulation only. The group 2 mice were treated with the Ribotan and antigen formulation. The group 3 mice received the antigen formulation only and the group 4 mice were the control, receiving only 0.2 ml of sterile 0.9% NaCl. After a 20 day regimen, the groups were inoculated with a dose of 750 ± 50 units of E.multilocularis protoscolexes per mouse.

After 90 days of incubation, the mice were euthanized and dissected for evaluation.

3 Results and discussion

Evaluation of the data of the experiment shows that all drugs used to stimulate the body's defensive response of mice against E.multilocularis with varying degrees of protective effect. Data of the experiment are presented in Table 2.

Table 2. Representative data for calculation evaluation the protective efficiency of immunization against secondary alveolar echinococcosis with Ribbon immunostimulation in combination an antigen from Echinococcus multilocularis protoscoleces

The formulations in 0.2 ml of sterile 0.9%NaCl per injection	*Number of group*	*Number of animals per group*	*Number of infected animals*	*Evaluation the protective efficiency of immunization*	*Protective efficiency*
ribotan (5 µl)	group 1	12	7	*The majority of mice infected in this group showed fertile parasite metacestodes.*	*41,7%*
antigen protein (60 µg)	group 2	12	5	*metacestodes with diameters of up to 2 mm were found, but there were no egg*	*58,3%*
antigen protein (60 µg) and ribotan (5 µl)	group 3	12	1	*singular metacestodes in the liver, with no infective egg elements*	*91,7%*
0.2 ml of sterile 0.9% NaCl.	group 4	12	12	*all mice were infected, showed fertile parasite metacestodes in this group*	*0 %*

The above table shows that the maximum protection against the Echinococcus multilocularis infection was obtained in mice immunized with the combination of antigen and Ribotan (91,7%). Only one mouse in this group showed singular metacestodes in the liver, with no infective egg elements. This was proved by a biological test on other mice.

The mice immunized with the protoscolex antigen only showed a protective effect of 58,3% In 5 mice from this group, metacestodes with diameters of up to two mm were found but there were no egg elements. The protective effect in the group treated with Ribotan only was less than 41,7%. The majority of mice infected in this group showed fertile parasite metacestodes.

The protective effect in the group treated with sterile 0.9% NaCl was 0%. All mice were infected in this group, showed fertile parasite metacestodes

Research results presented below graphically.

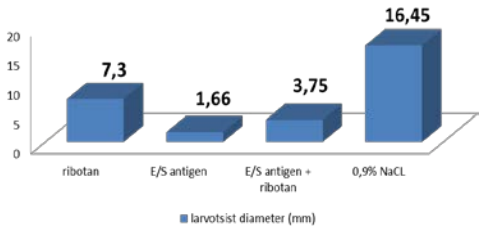


Fig. 3 The results of measurement dimensions of metacestodes of secondary alveolar echinococcosis on 90 days post infection (p.i.), in mm

Figure 3 shows the results of measurements parasite cysts size in millimeters.

We can see that the largest diameter metacestodes achieved in the fourth control group received of sterile 0.9% NaCl. In the first group of mice that received the experimental treatment using "Ribotan" metacestod's diameter was in 2.25 times less than in metacestodes in the control group. Metatsetod's diameter in the second and the third groups of mice was 9.91 and 4.39 times less than the control group, respectively.

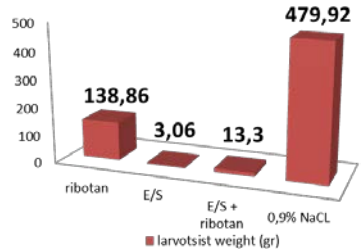


Fig. 4 The results of measurement of larvotsist's weight in experimental secondary echinococcosis on 90 days after infection in grams.

The above figure 4 presents the results of measurements of larvotsist's weight in grams. We can see that the maximum weight of larvotsist achieved as in the fourth control group receiving saline. Larvotsist's weight in the first group (receiving only immunomodulator) was 3.45 times lower than in the control group. Flyweight larvotsist observed in the second group receiving excretory-secretory antigen in a dose of 60 µg of protein per mouse. Larvotsist's weight in the third group (treated with a complex formulation) was 36.03 times less than in the control group.

However, only weight and size don't give a complete picture of the protective protection products. The presence of pathogenic elements in secondary larvotsists that can cause infestation in the secondary - intermediate hosts is one of the most important factors in evaluating the protective effect of complex immunization formulations. Infective material obtained in the course of the experiment from the experimental mice we studied by bioassay in other laboratory mice.

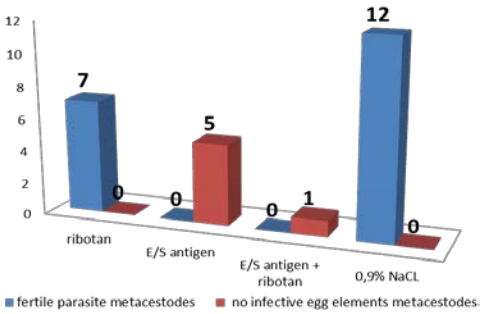


Fig. 5 Comparative results between the presence of mature pathogens and non-invasive elements in metatsestodes in secondary experimental echinococcosis.

Figure 5 presents results of the comparison between the presence of invasive pathogens and undeveloped elements in metatsestodes in the secondary echinococcosis. There are significant differences between metatsestodes with no infective egg elements and fertile parasite metacestodes. The greatest positive influence of immunoprophylaxis showed complex application excretory-secretory antigen (60 µg) and Ribotan (5 µl). In this group (group 3) only one mouse developed singular metacestodes in the liver, which didn't contain infective egg elements. The use of the E-S antigen had a positive effect too. The number of animals having developed larvotsists was higher in this group than in the group 3. But these metatsestods were small and didn't contain the same elements invasive. Application immunomodulatory ribotan (experimental group one) led to a decrease in the number metatsestods, but all the elements were fertile and invasive. But the highest number of mature pathogens developed in group 4 (control). All mice were infected in this group, all showed fertile parasite metacestodes.

The main objective of this work is evaluation the protective efficiency of immunization against secondary alveolar echinococcosis, with an antigen from Echinococcus multilocularis protoscolexes in combination with Ribotan immunostimulation.

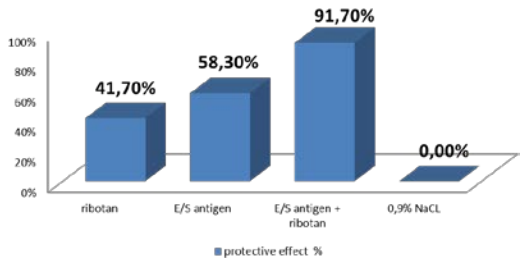


Fig. 6 protective efficiency of immunization against secondary alveolar echinococcosis.

Figure 6 presents results of evaluation the protective efficiency of immunization against secondary alveolar echinococcosis. We can see that the maximum protective effect was achieved in the third group in the with application an antigen from Echinococcus multilocularis protoscolexes in combination with Ribotan immunostimulation.

Application of Ribotan or E-S antigen separately, didn't show strong protective effect.

4 Conclusion

Our experiment showed that the use of immunostimulators in combination with specific antigens for the immunoprophylaxis against secondary alveolar echinococcosis has a synergistic effect on the treatment. Thus, in all the experiments it was clearly demonstrated the advantage of the drug complex comprising the specific antigen and immunostimulating agents, to achieve the best protective effect.

Our data are actually not inconsistent with the results of other researchers which have used various immunomodulating and adjuvant agents. That enhance the immunogenicity of antigens as a means of increase potentiation animal body defense mechanisms against helminthiasis [12, 6, 7, 16, 18].

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