Influence of azoxymer bromide on the cellular composition of the spleen in experimental infection of T. spiralis

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Abstract. The experiment used white mice, which were divided into 4 groups. The first group of mice was injected with a saline solution with 0.04 mg of Trichinella antigen obtained by the Magat method subcutaneously, the second group of mice was injected with azoxymer bromide (polyoxidonium) at a dose of 0.004 mg/mouse, the third group was infected with Trichinella larvae without preliminary administration of drugs, group 4 served as a control. A week after the last injection of drugs, mice were infected with invasive Trichinella larvae at a dose of 40 larvae/animal. Evaluation of the protective properties of polyoxidonium in laboratory models was carried out by determining the intensity of invasion in studies of all muscle groups of the animal by the method of peptolysis and histological material to assess the cellular composition of the spleen. The protective effectiveness of polyoxidonium was 99.43%. The rearrangement of the structure of the spleen under the influence of an immunostimulant indicated a high degree of reactivity of the spleen of animals in response to the introduction of polyoxidonium (the ratio of red and white pulp and their cellular composition changes).

1. Introduction

Trichinosis is a dangerous helminthiasis in humans and animals caused by helminths: Trichinella spiralis (spiralis), Trichinella spiralis (nativa) and Trichinella pseudospiralis, widespread in the Russian Federation [1,2]. Experimental reproduction of this helminthiasis is one of the most effective models for pharmacological, parasitological, immunological and general biological studies [3].

Considering that the interest in the use of immunostimulating drugs is increasing every year both in treatment and prevention work and among scientific research. Parasitological immunology is no exception: the use of immunostimulants increases the reactivity of the host organism in relation to specific parasitic antigens and enhances the body's resistance, in some cases this leads to a decrease and even to the complete prevention of helminth infection [1,2].

In addition, drugs with immunotrophic activity, in therapeutic doses, restore the coordinated work of the parts of the immune system, the functioning of which, as a rule, is imbalanced in helminthiases,
many of them are also antioxidants. Immunostimulants began to be used in helminthology only in the last decades, so there is still no single point of view on the appropriateness of their use [4]. However, a number of drugs, such as levamisole, interferon inducers, are successfully used in helminthology.

Until now, the use of immunostimulants in the treatment of trichinosis is poorly studied and fragmentary. Single attempts to introduce them into treatment regimens often gave side effects, which, apparently, was due to the long persistence of the parasite in the body, and, consequently, to the high antigenic load. However, over the past decade, the understanding of the immune system has deepened and new targeted drugs have appeared that stimulate its specific links. So, for example, a number of cytokines are successfully used in modern parasitology, mainly synthetic compounds, their inducers.

Interferons are quite promising immunostimulants in parasitology, used for prevention and treatment. In the treatment and prevention of trichinosis, the following drugs belonging to different groups were used: synthetic - such as azoxymer bromide (polyoxidonium), roncoleukin (recombinant IL2) and immunostimulants of biological origin - sodium nucleinate, crucine prodigiosan, subalin, ribomunyl, and others. The above drugs, etc. were tested in the Russian Federation and abroad on experimental models in laboratory conditions for trichinosis in laboratory animals (mice and rats) in treatment and prevention regimens. The ability of immunostimulants to enhance the functional activity of neutrophils and macrophages in helminthiasis was reliably noted.

For example, Martinez-Gomez F. et all [4] indicate that oral administration of Lactobacillus casei for 6 weeks induces complete protection against Trichinella infection, however, the doses of larvae administered by the experimenters were very small.

The protective effect of the immunomodulator roncoleukin (within 40%) was established in canine echinococcosis and in laboratory studies in Wistar rats with trichinosis in conventional doses of infection. There are also data on the protective effect of the immunomodulators azoxymer bromide, cycloferon and roncoleukin in experimental trichinosis in laboratory outbred white mice [5-7]. Also, on the model of experimental alveolar echinococcosis, the protective effect of the immunostimulating drug ribotan in combination with the cellular antigen of the protoscolex Echinococcus multilocularis was established, and the use of immunofan as an immunotherapeutic agent in combined immunotherapy in the treatment of echinococcosis was very successful [6].

2. Materials and methods
The research was carried out on the basis of the VNIIP center - a branch of the Federal Research Center - VNIIIEV named after K.I. Scriabin. The experiment used 40 white mice, which were divided into 4 groups of 10 animals in each group.

The first group was injected with a saline solution with 0.04 mg of Trichinella antigen obtained by the Magat method (1976) [8] subcutaneously, the second was injected with azoxymer bromide (polyoxidonium) at a dose of 0.004 mg/mouse, the third group was infected with Trichinella larvae without preliminary administration of drugs, 4 group served as a control.

A week after the last administration of the preparations, mice of groups 1-3 were infected with invasive Trichinella larvae at a dose of 40 larvae/animal, excluding the control group. Evaluation of the protective properties of polyoxidonium in laboratory models for trichinosis was carried out by determining the intensity of invasion during postmortem studies of all muscle groups of the animal. For this purpose, all mice were slaughtered after 60 or more days, their muscle mass was subjected to digestion in artificial gastric juice (IGS), each mouse separately and the number of Trichinella larvae was counted. Simultaneously with the removal of the animals from the experiment at the indicated time, the histological material (spleens) was taken for histomorphological evaluation.

The material was fixed in a 10% neutral aqueous formalin solution. Paraffin wiring of biological material was carried out at the V.I. prof. B.M. Zhitkov. After fixation in formalin, fragments of 1x1 cm³ were cut out from the prepared biomaterial, according to the classical technique (GA Merkulov, 1969) [9], and it was guided, embedded in paraffin, histological sections 5-7 μm thick were prepared and stained with hematoxylin and eosin and azure II - eosin according to Nekhta-Maksimov modified by
Merkulov (1969) [9]. The total number of white and red pulp cells was counted: the percentage of different lymphocytes and plasma cells was calculated separately.

Calculation of the cellular composition on each histological specimen was carried out in 50 fields of view, selected by arbitrary movement of the specimen, using an ocular measuring grid for cytostereometric studies proposed by G.G. Avtandilov (1991) [10], on a microscope MBI - 3U42, objective 100/1.25.OIL, eyepiece WF 10x18. Determination of the size of individual cells, their refinement differentiation, morphometry, and microphotography were performed using a VisionBio microscope (Epi) with automatic display. The identification of cells (macrophages and leukocytes) was carried out with a magnification of the objective x 20 and x100 according to GS. Katinas (1981) [11].

Separately, the percentage of cells of the lymphocytic lineage was deduced: medium lymphocyte, large lymphocyte, small lymphocyte, plasmablast, plasmacyte. The experiments were carried out in accordance with the Declaration of Helsinki on the humane treatment of animals, the principles of humanity set out in the directive of the European Community (86/609/EC), "Rules for conducting work using experimental animals" (1977), "Bioethical rules for conducting research on humans and animals "(2004) [12].

3. Results and discussion

Protective efficacy with the introduction of antigen in experimental trichinosis was 62.9%, the number of Trichinella larvae was 750±110, polyoxidonium 99.43%, 57±20 Trichinella larvae/per animal were found in the entire muscle mass. In group 3 (without immunostimulation), the number of larvae was 2246±210.

The next stage of the assessment was the study of the effect of immunostimulation on the lymphoid tissue of the spleen in the study of histological preparations compared the reactive processes occurring in the spleen in the studied groups of animals. In all experimental animals, a restructuring of the spleen structure under the action of immunomodulators was noted, which was of a rather uniform nature. The greatest changes were noted in the ratio of the area of red and white pulp, and their cellular composition.

Under the action of antigenic stimulation by growing Trichinella larvae in all groups of infected mice (1,2,3), white pulp follicles grow, merging into large conglomerates of lymphoid tissue, consisting of 4-7 former nodules (figure 1).

Figure 1. Large conglomerates of lymphoid tissue in the red pulp stained with Azur II - eosin according to Nekhta-Maksimov modified by Merkulov (1969), lens magnification x20.

Accumulations of lymphocytes are also observed in the red pulp, against the background of few erythrocytes and stromal elements. During differentiation of lymphocytes (x100 magnification), the following groups were distinguished:
Lymphocytes are small, usually make up the majority of peripheral blood lymphocytes. Their diameter is 6-10 μm, the nucleus is round, rarely slightly oval, dark-colored, with dense chromatin, occupies most of the cell. Large, rounded nuclei of small lymphocytes are evenly surrounded by a narrow rim of cytoplasm. In the nuclei, compact heterochromatin is well visualized; at high magnification, the nucleolus is also visible. The cytoplasm is visible as a very narrow rim. In the sections, light and dark small lymphocytes with different densities are determined. The average lymphocyte is a mature cell of the lymphoid series, usually 10-12 μm in size, with a round, oval nucleus, and consists of coarse formations of chromatin resembling lumps. The nucleus is stained dark violet, the cytoplasm is light blue with enlightenment around the nucleus. The lymphocyte is large, the diameter of which is from 12 to 16 μm, the cytoplasm occupies a significant part of the cell, light blue. Nuclear chromatin is less dense than that of other lymphocytes.

A group of plasma cells was isolated separately, including:

- **Plasmablasts** - cells with a size of 16-23 μm, have a nucleus of a delicate structure, which occupies most of the cell, being located centrally or somewhat eccentrically. The cytoplasm is large enough, bright blue, with a characteristic perinuclear zone of enlightenment.

- **Proplasmacytes** are transitional forms from plasmablast to mature plasmacyte. The cell size is slightly larger than that of a mature plasma cell (sometimes up to 20 μm). The nucleus occupies most of the cell and is often eccentrically located. The cytoplasm is sharply basophilic with enlightenment around the nucleus. Plasma cells are mature plasma cells. They are very diverse in shape and size (size from 8 to 20 μm). The nucleus is round with a coarse wheel-like striation, eccentrically located. The cytoplasm is intensely blue with a pronounced perinuclear zone of enlightenment; contains vesicles and vacuoles.

Sometimes there are two- and three-core forms. Macrophages have a characteristic irregular shape and large size, which makes it easy to distinguish them from other cells of the white pulp of the spleen. These cells are significantly predominant in the groups of infested mice, more often found in the red pulp (figure 2).

**Figure 2.** Spleen macrophages stained with Azur II - eosin according to Nekhta-Maksimov modified by Merkulov (1969), lens magnification x100.

When counting the cells of the lymphoid series of large lymphocytes in both the red and white pulp in the group of mice that were injected with polyoxidonium, compared to the administration of antigen,
there were 1.5-2 times more (P <0.01), and small lymphocytes were also 1.5 times less (P <0.01). The number of cells with mitotic figures in mice with polyoxidonium in the red pulp was 2.3 times higher and 4 times higher in the white pulp (P <0.001) (table 1).

**Table 1. Changes in the cellular composition of the spleen during immunostimulation.**

| Antigen          | Lymphocytes (red pulp) | Lymphocytes (white pulp) | Mitosis       | Plasmocytes |
|------------------|------------------------|--------------------------|---------------|-------------|
|                  | average | large  | small | average | large | small | red pulp | White pulp | red | white |
| Polyoxidonium    | 65.3±   | 4.3±   | 23.6± | 52.3±   | 4.6±   | 33.0± | 4.3±   | 2.6±   | 7.0± | 5.0±   |
|                  | 3.5     | 0.41   | 1.08b | 7.3     | 0.41   | 1.0b  | 0.82   | 0.82   | 0.7  | 0.7    |
|                  | 0.4     | 0.41b  | 0.71  | 0.41    | 0.41   | 0.41c | 0.41c  | 0.41c  | 0.7  | 0.4    |

Note: differences between groups are significant *P<0.05, b - P<0.01, c- P<0.001

4. Conclusions
A significant protective activity of polyoxidonium (99.43%) was established in parasitological studies. When studying histological preparations, it was noted that the restructuring of the spleen structure under the influence of immunomodulators is of the same type: the ratio of the red and white pulp, their cellular composition changes. The follicles of the white pulp grow, merging into large conglomerates of lymphoid tissue. The results obtained indicate a high degree of reactivity of the spleen of animals in response to the introduction of polyoxidonium. Therefore, polyoxidonium can be used as an immunostimulating agent both in the treatment and in the specific prevention (vaccination) of trichinosis along with fractionated and excretory-secretory antigens of *Trichinella spiralis* [13, 14, 15, 16].

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