

Influence of Anti-tuberculosis Drug KIM-M2 on Morphology of Lymph Nodes, Spleen, Liver and Lungs of Guinea Pigs Infected with *M. bovis*

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Abstract—Conjugation of immunogenic fraction separated from microbial cells of BCG strain with stimulating component proved to be a promising direction in the creation of anti-tuberculosis drugs. One of such components is KIM-M2, a complex of thermostable soluble antigens extracted from microbial cells of BCG strain incubated with formalin and conjugated with polyvinyl pyrrolidone and polyethyleneglycol. To study the impact of KIM-M2 on the morphology of lymph nodes, spleen, lungs and liver of guinea pigs, the animals of group 1 (control, $n = 5$) were injected subcutaneously with a virulent culture of *M. bovis* strain 8 at a dose of 0.001 mg/ml. Animals of group 2 ($n = 5$ subjects) were injected subcutaneously with KIM-M2 at a dose of 0.5 mg/ml of protein, then 30 days later they were injected with a virulent culture of *M. bovis* strain 8, subcutaneously at a dose of 0.001 mg/ml. Animals of group 3 ($n = 5$ subjects) were injected subcutaneously with a virulent culture of *M. bovis* strain 8 at a dose of 0.001 mg/ml, 14 days later they were injected with KIM-M2 at a dose of 0.5 mg/ml of protein. Histomorphologic changes in animals of group 1 can be considered as morphological equivalent of immune suppression reaction. At the same time, the animals from group 2 and especially from experimental group 3 showed an increase of immunogenesis that can be proved by a profound proliferation of lymphocytes and macrophages.

Keywords—cattle, anti-tuberculosis drug KIM-M2, histomorphology, organs.

I. INTRODUCTION

Bovine tuberculosis remains a serious problem for human and animal health all around the world, thus we require some new means to combat this disease [1-7]. Over the past 20 years, significant progress has been made in designing and assessing anti-tuberculosis vaccines for cattle with new attenuated mycobacterial strains, providing an alternative for BCG vaccine and subunit vaccines [2, 8-10]. The most promising concept of designing anti-tuberculosis drugs is conjugation of an immunogenic fraction extracted from the vaccine bacteria with a stimulating component. As results of the research have shown, synthetic unnatural polyelectrolytes with controlled structure have all the necessary properties [11]. Such vaccines include conjugates prepared on the basis of BCG and synthetic polyelectrolytes, in particular, immunomodulator KIM-M2, which has corresponding protective properties [11].

All organs of the body histiocytic system are involved into the pathologic process due to various forms and stages of tuberculosis with the subsequent formation of granulomatous inflammation. However, there is not enough research concerned with pathomorphological, and particularly with histological changes, developing in organisms of animals suffering from tuberculosis. Moreover, the study of tissues and organs infected with mycobacteria considering the effect of subsequently injected anti-tuberculosis drug KIM-M2, developed by us and being a complex of thermostable soluble antigens extracted from microbial cells of BCG strain incubated with formalin and conjugated with polyvinyl pyrrolidone (PVP) and polyethyleneglycol (PEG), can be very interesting both in theoretical and practical perspective.

It was previously shown that KIM-M2 restores impaired immune responsiveness effectively, eliminates secondary immunodeficiencies, has expressed protective properties and is harmless to animals. However, there is not enough research on the morphological changes of organs in animals experimentally infected with *M. bovis* in the course of using this antigen-polymer complex.

II. MATERIALS AND METHODS

The research was conducted in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [12]. We used a virulent culture of *M. bovis* strain 8 for experimental infection of guinea pigs. The complex of antigens for conjugation with polyelectrolytes was isolated from the culture of BCG vaccine strain, which was grown on liquid synthetic Sauton's medium, then subjected to ultrasonic disintegration using UZDN-1 ultrasonic disperser (22 kHz, 60-70 W/cm² for 30 min). The resulting suspended matter was centrifuged, then supernatant protein content was determined using biuret test.

The research was conducted on 15 mature guinea pigs; they were split into 3 experimental groups, 5 animals in each group. Animals of group 1 (control, $n = 5$) were injected subcutaneously with a virulent culture of *M. bovis* strain 8 at a dose of 0.001 mg/ml. Animals of group 2 ($n = 5$) were injected subcutaneously with KIM-M2 at a dose of 0.5 mg/ml of protein, then 30 days later they were injected with a virulent culture of *M. bovis* strain 8, subcutaneously at a dose of 0.001 mg/ml. Animals of group 3 ($n = 5$) were

injected subcutaneously with a virulent culture of *M. bovis* strain 8 at a dose of 0.001 mg/ml, 14 days later they were injected with KIM-M2 at a dose of 0.5 mg/ml of protein. 45 days after infection contamination, the animals were sacrificed by decapitation.

Histological studies of lungs, spleen, liver and inguinal lymph nodes autopsies were performed. Histologic material was fixed in formalin solution, neutral phosphate buffered, 10%. Paraffin embedding was performed using paraffin embedding center MICROM ES-350. Histologic sections 5-7 micron thick were made by rotary mikrotome MICROM NM 340. Histologic sections were painted with haematoxylin and eosin and then were microscopied using microscope Zeiss AXIO Imager A1.

III. RESULTS AND DISCUSSION

It has been established that typical tuberculous granulomas were formed in the inguinal lymph nodes of guinea pigs infected with *M. bovis* (group 1), accompanied by a diffuse proliferation of stroma cells and fibrotisation of cortical substance (Fig.1). In most cases granulomas were on various stages of development.

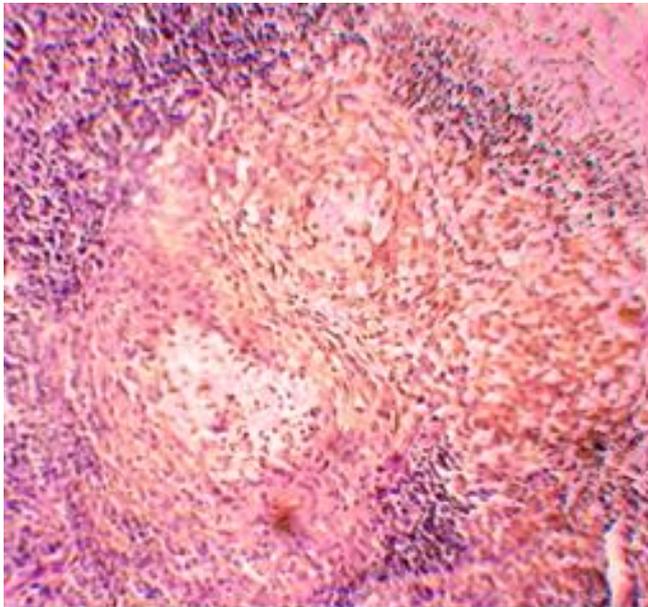


Fig. 1. Tuberculosis granulomas in the cortical substance of the inguinal lymph nodes of guinea pigs from Group 1. Haematoxylin and eosin staining, magnification: 1x10.

There are usually epithelioid macrophages in the central part of granulomas, these are large cells with pale eosinophilic cytoplasm and bright large nucleus. Some of them have signs of cytolysis, nuclei are missing, cytoplasm is vacuolated. A small number of granulocytes appear in the emerging necrotic area of granulomas. There are also epithelioid macrophages without any signs of necrobiosis or necrosis around this area. We can also observe some fragments of phagocytized nuclei in the cytoplasm of epithelioid macrophages. In most cases, the outside area of epithelioid cells is surrounded by a ring of fibroblasts and fibrocytes. In some granulomas the cytoplasm of epithelioid macrophages contains a large number of vacuoles which makes it look foamy. At the same time, we did not observe any signs of mineralization in the emerging granulomas of cortical substance in inguinal lymph nodes of guinea pigs

from group 1. In the inguinal lymph nodes, there was an expansion of the medullary sinuses (Fig. 2).

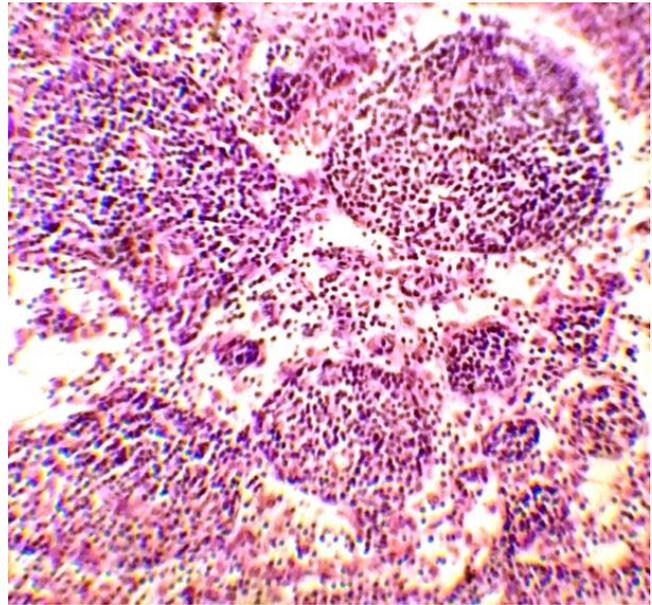


Fig. 2. The medulla of the inguinal lymph node of a guinea pig from Group 1. The medullary sinuses are expanded, they are filled with rarely located cell populations. Haematoxylin and eosin staining, magnification: 1x10.

At the same time, the number and the size of lymph follicles was decreased, the level of lymphogenesis, as well as the number of plasmocytes in medullated strings and brain sinuses were reduced that can be a morphological evidence of immunosuppressive effect of the causative agent of tuberculosis. Morphological signs of decreasing activity of lymph follicles, increased proliferation of reticulocytes and macrophages in red pulp cords were found in the spleens of guinea pigs from group 1. Thus, we found that there was no clear boundary between the germinal centers and surrounding cells in the spleen lymph follicles of guinea pigs from this group. At the same time, we could scarcely observe dendritic reticulocytes in these germinal centers, in most of the cases they show morphological signs of necrosis. It should be noted that among reticulocytes there are also macrophages with phagocytized remnants of nuclei. Germinal centers of follicles, as well as inguinal lymph nodes there proved to be a rare location of lymphocytes. Some lymph follicles of spleen of animals from group 1 had a pronounced swelling of adventitia and their arteries as well as mucoid degeneration of the walls of these blood vessels. We also recorded a small quantity of mature plasmocytes and a large number of granulocytes in the cords and sinuses of the spleen. In the red pulp of the spleen we found expansion of sinuses. In their lumens we detected macrophages with phagocytized erythrocytes, as well as a small number of plasmocytes. We believe that these changes might be explained by the specific effect mycobacteria of bovine tuberculosis cast upon the bodies of experimental animals. In the lungs of the animals of group 1, we found a proliferative activity of the stroma, which leads to the thickening of interalveolar septa and creates the foci of necrosis involving bronchial walls. In the liver, we found the accumulation of mononuclear cells around blood vessels and bile duct triads.

In the lungs of guinea pigs from group 1 infected with *M. bovis*, we detected an intensive proliferation of macrophages both in peribronchial connective tissue and in the walls of pulmonary alveoli. At the same time, thickening of alveolar walls on the periphery of lung tissue was combined with development and in some cases with ectasia of small bronchi. Across the surface of histologic sections of peripheral part of lobes of animals' lungs, we observed the groups of scattered lymph follicles of different sizes. Analysis of serial histologic sections revealed a close relationship between lymph follicles and blood vessels of different diameters. In an initial stage of development of follicle cells, lymphocytes surround a blood vessel in the form of a coupling. Whereas the further growth of lymphoid tissues develops eccentrically with the formation of typical lymph follicles.

The major cell populations of thickened alveolar septums are cells with oxyphilic sometimes vacuolated cytoplasm. Morphologically these proliferating cells are similar to epithelioid macrophages but we cannot exclude that in some cases these may be alveolar macrophages. In most thickened interalveolar septums, we detected lymphocytes and a few granulocytes scattered between macrophages. Proliferative processes in lung tissue of guinea pigs from group 1 were expressed in the area of major branches of bronchi most significantly. In most areas of histologic sections, these bronchi, as well as their accompanying blood vessels, were surrounded by solid margins of proliferating macrophages, among which we detected large lymph follicles and areas infiltrated by lymphoid cells. Lymph capillaries in these areas were dilated. We also detected swelling of perivascular and peribronchial connective tissues, and in some cases we observed mucoid degeneration thereof. At the same time, we observed lysis of major bronchus wall in one area of peribronchial connective tissue swelling, thus the lumen of the bronchus touched the surrounding tissue. It is interesting that there is no accumulation of granulocytes either in the content of the bronchus or in the area of the bronchus destroyed. In addition, we did not find any granulomas typical for tuberculosis.

The analysis of pathological changes in guinea pigs infected with *M. bovis* (Group 1) showed that on the 45th day after introduction of the infection, the frankest specific changes were developed in the inguinal lymph nodes, into which the causative agent of tuberculosis penetrated predominantly through lymph. The specific granulomas started to form in the inguinal lymph nodes. Also, we detected the diffuse proliferation of macrophages and fibroblasts. We observed that the immunosuppressive activity of the pathogen was revealed in the decrease in the number and size of lymphatic follicles, in the retrogression of lymphogenesis, as well as in the decrease in the number of plasma cells in the pulpy cords and sinuses of the medulla. In the spleen, we detected morphological signs of decreased lymphatic follicles, increased proliferation of reticulocytes and macrophages in the pulpy cords. In the lungs, along with the proliferative activity of macrophages of the stroma, there were necrotic sections with involved bronchial walls in the process, which was the initial stage of the formation of cavities. At the same time, specific granulomas were not found.

In the liver of guinea pigs infected with *M. bovis* and subjected to slaughter on the 45th day after infection, we saw accumulations of mononuclear cells composed mostly of lymphocytes around blood vessels of liver triads. In some areas, populations of mononuclear cells formed clusters around bile ducts. We observed a large number of granulocytes with acidophilic stippling in venous vessels. In some areas of the liver of animals from this group we detected granular degeneration of hepatocytes.

In the inguinal lymph nodes of animals from group 2, we saw an intense proliferation of macrophages and stromal cells in the cortex and medullary substance. Besides, we observed vast areas in the cortical substance that did not have any follicular structure. In the spleen of guinea pigs from group 2, in contrast to groups 1 and 3, there was much larger quantity of lymph follicles. Follicles stand out against the red pulp less clearly because it contains a large quantity of lymphocytes. Proliferative processes in spleen cord were less pronounced than those of group 1. In the lungs of guinea pigs infected with *M. bovis* in 30 days after injection of KIM-M2 and subjected to slaughter on the 45th day after infection, we noted a number of pathohistological changes other than in animals from groups 1 and 3. Unlike guinea pigs from group 3, they showed a consistent diffuse thickening of alveolar walls as a result of the active proliferation of alveolar macrophages, the same as in the animals of group 1 (Fig.3).

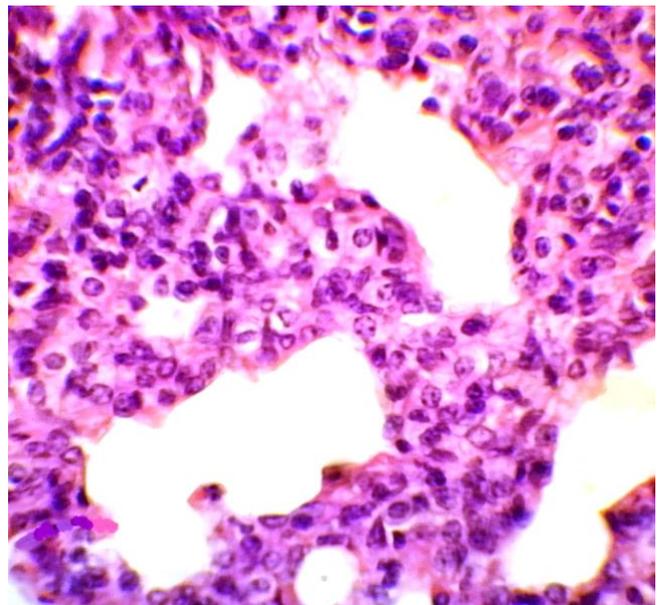


Fig. 3. The lung of a guinea pig from Group 2. Thickening of alveolar walls due to proliferation of macrophages. Haematoxylin and eosin staining, magnification: 1x10.

At the same time unlike guinea pigs from groups 1 and 3, they did not show any noticeable proliferation of macrophages and lymphocytes in the areas of bronchial branches with large diameters.

In the liver of guinea pigs from group 2, as well as in animals from group 3, we detected clumps of monocytes consisting predominantly of lymphocytes around blood vessels and bile ducts of some liver triads (Fig. 4).

The liver also showed granular and fatty degeneration of hepatocytes, especially in liver cells under the capsule, in the organ itself and around the central vein. In the lungs of guinea pigs from groups 2 and 1, we found the proliferation of epithelioid macrophages in the alveoli walls, as well as in perivascular and peribronchial connective tissues of respiratory tract. We also registered some lymph follicles formed in respiratory tract.

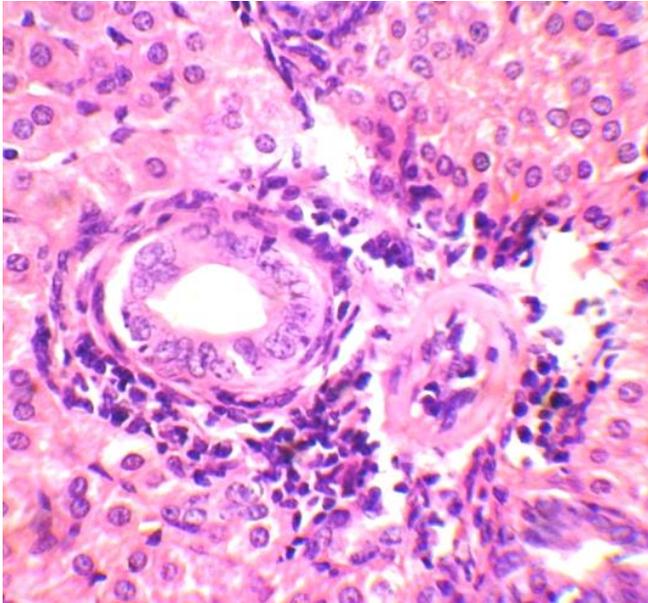


Fig. 4. The liver of a guinea pig from Group 2. Around the blood vessels and bile ducts, there is concentration of mononuclear cells, consisting mainly of lymphocytes. Haematoxylin and eosin staining, magnification: 1x10.

In the cortical substance of inguinal lymph nodes of guinea pigs from group 3, in contrast to the animals from group 1, we found a large quantity of lymph follicles. In some areas, as a result of an increase in the size of follicles and their quantity, they merged and fully filled the cortical substance. In the spleen sinuses of the animals from group 3, we detected a large quantity of granulocytes. In some parts of the red pulp of the spleen, histoarchitectonics was damaged due to necrotic processes.

In the lungs of the animals from group 3, compared to guinea pigs from group 1, we saw a less pronounced proliferation of macrophages in the alveoli walls and in perivascular and peribronchial connective tissues.

At the same time, unlike the lungs of guinea pigs from Group 1, large parts of induration and necrosis in the area of the branch of the large bronchi were not found.

In the liver of the guinea pigs from group 3, we found a slight proliferation of mononuclear cells around blood vessels and small bile ducts. In the lumens of the capillary, we detected a large number of granulocytes.

In addition, there were no specific granulomas in the liver of guinea pigs from Group 3 as well as Group 1 and 2.

IV. CONCLUSION

In the course of histomorphologic research, we found that typical tuberculosis granulomas were formed in inguinal lymph nodes of guinea pigs infected with a virulent culture

of *M. bovis* (group 1). This process was accompanied by a diffuse proliferation of stromal cells and fibrocyte of the cortical substance of the organ. At the same time, the animals from groups 2 and 3 that were injected with KIM-M2 did not show any tuberculosis granulomas.

In the spleen of the animals infected with a virulent culture (group 1), compared to the animals of groups 2 and 3, we observed morphological signs of decreasing activity of lymph follicles in medullated cords. The pathological changes found serve as morphological evidence of immunosuppressive effect of the causative agent of tuberculosis.

By the 45th day, immune response to the pathogen developed in the lungs of guinea pigs from group 1 which manifested itself in perivascular proliferation of lymphocytes and formation of typical lymph follicles.

We did not find any specific granulomas in the liver of guinea pigs from groups 2 and 3. At the same time, in animals from these groups, we registered little proliferation of mononuclear cells around blood vessels and bile ducts of small diameter.

Analysis of pathologic changes in the inguinal lymph nodes, spleen, lungs and liver suggested that guinea pigs infected with *M. bovis* and treated with antituberculosis drug KIM-M2 did not show formation of specific granulomas, compared to the animals infected with this virulent culture but not injected with KIM-M2. At the same time, the animals of groups 2 and 3 showed increased immunologic restructuring of their organisms with the injection of KIM-M2 which led to less severity of the pathologic process after experimental infecting with the causative agent of tuberculosis. In view of the above, we believe that KIM-M2, in doses indicated, can be used as a specific anti-tuberculosis medication for the treatment of infection caused by *M. bovis*.

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