FEATURES OF THE PATHOGENETIC MECHANISMS OF TUBERCULOUS PERITONITIS IN AN EXPERIMENT

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The prevalence of tuberculous peritonitis that has been observed in the recent decades is the result of lymphohematogenous spread of Mycobacterium tuberculosis (MBT) from lungs and other extrapulmonary sources. It is still unclear why certain organs and anatomical regions get involved in the inflammatory process during generalization of the tuberculosis infection. Why do some cases develop into peritoneal tuberculosis and other into kidney tuberculosis? Thus study aimed to investigate the pathogenesis of tuberculous peritonitis in a reproducible biological model. Tuberculous peritonitis was modeled in 18 rabbits (10 in the test group, 8 in control) by intraperitoneal inoculation of the MBT suspension. In order to suppress peritoneal macrophages and major cytokines, test group rabbits were injected with the TNFα inhibitor and iron (III) hydroxide sucrose complex before being infected, while control group rabbits received no immunosuppressive drugs. Autopsy of the control group animals revealed changes characteristic of pulmonary tuberculosis in 37.5% of cases, with no damage to other organs and systems registered. Conversely, test group rabbits had the signs of tuberculous peritonitis in their abdominal cavities. The results of this study suggest that it is the local immunity of an anatomical area that largely determines whether a secondary focus of extrapulmonary tuberculosis infection will develop there or not. For the peritoneum, a smaller pool of peritoneal macrophages and weaker cytokine production is a necessary and sufficient condition to have tuberculous peritonitis developing therein.

Keywords: peritonitis, animal model, tuberculous peritonitis, abdominal tuberculosis, TNFα, tumor necrosis factor, rabbit

Conflict of interests: Plotkin DV, Vinogradova TI, Reshetnikov MN, Zyuzya YuR, Sinitsyn MV, Bogorodskaya EM, Yablonsky PK have filed a request to the Federal intellectual property service "Federal Institute for Industrial Property" to register the Russian Federation patent for the invented "Method for modeling tuberculous peritonitis" (registration #2021114954 of May 25, 2021). Ariel BM, Zhuravlev VYu claim to have no conflict of interest.

Author contributions: Plotkin DV, Vinogradova TI, Sinitsyn MV, Bogorodskaya EM — study concept and design development; Plotkin DV, Reshetnikov MN, Zhuravlev VYu, Zyuzya YuR — material collection; Reshetnikov MN, Zhuravlev VYu — statistical processing; Plotkin DV, Vinogradova TI, Ariel BM, Yablonsky PK — analysis of the data obtained; Plotkin DV, Ariel BM, Sinitsyn MV, Bogorodskaya EM — text preparation; Vinogradova TI, Zyuzya YuR, Yablonsky PK — editing.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Saint Petersburg Research Institute of Phthisiopulmonology (protocol № 73 of December 23, 2020). All manipulations with animals conformed to the requirements of the European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes (CETS # 170) and followed guidelines provided in GOST 33216-2014 Rules for Working with Laboratory Rodents and Rabbits.

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Received: 30.06.2021 **Accepted:** 21.07.2021 **Published online:** 04.07.2021

DOI: 10.24075/brsmu.2021.036

ОСОБЕННОСТИ ПАТОГЕНЕТИЧЕСКИХ МЕХАНИЗМОВ ТУБЕРКУЛЕЗНОГО ПЕРИТОНИТА В ЭКСПЕРИМЕНТЕ

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Наблюдаемый в последние десятилетия рост числа случаев туберкулезного перитонита обусловлен лимфогематогенным распространением микобактерий туберкулеза (МБТ) из легких и других экстрапульмональных источников. До сих пор остается неясным, почему при генерализации туберкулезной инфекции в воспалительный процесс вовлекаются те или иные органы и анатомические области. Почему в одних случаях развивается туберкулез брюшины, а в других туберкулез почек? Целью работы было изучить патогенез туберкулезного перитонита с помощью создания воспроизводимой биологической модели. Туберкулезный перитонит моделировали на 18 кроликах (10 особей — экспериментальная группа, 8 — контрольная) путем внутрибрюшинной инокуляции взвеси МБТ. Кроликам экспериментальной группы перед заражением вводили ингибитор TNF — и железа (III) гидроксид сахарозный комплекс с целью подавления активности перитонеальных макрофагов и основных цитокинов; в контрольной группе введение иммуносупрессивных препаратов не производили. При аутопсии животных контрольной группы в 37,5% случаев выявлены изменения, характерные для туберкулеза легких, поражения других органов и систем не отмечено. Напротив, у кроликов экспериментальной группы в брюшной полости выявлены признаки туберкулезного перитонита. По результатам проведенной работы, анатомическая область, где развивается вторичный очаг внелегочной туберкулезной инфекции, во многом зависит от местной иммунной защиты. Так, для брюшины таким необходимым и достаточным условием развития туберкулезного перитонита являются уменьшение пула перитонеальных макрофагов и снижение цитокиновой продукции.

Ключевые слова: перитонит, животная модель, туберкулезный перитонит, абдоминальный туберкулез, TNFα, фактор некроза опухолей, кролик

Конфликт интересов: Д. В. Плоткин, Т. И. Виноградова, М. Н. Решетников, Ю. Р. Зюзя, М. В. Синицын, Е. М. Богородская, П. К. Яблонский — подали заявку в Федеральную службу по интеллектуальной собственности «Федеральный институт промышленной собственности» на регистрацию заявления о выдачи патента Российской Федерации на изобретение «Способ моделирования туберкулезного перитонита» (регистрационный № 2021114954 от 25 мая 2021 г.). Б. М. Ариэль, В. Ю. Журавлев — заявляют об отсутствии у них конфликта интересов.

Вклад авторов: Д. В. Плоткин, Т. И. Виноградова, М. В. Синицын, Е. М. Богородская — разработка концепции и дизайна исследования; Д. В. Плоткин, М. Н. Решетников, В. Ю. Журавлев, Ю. Р. Зюзя — сбор материала; М. Н. Решетников, В. Ю. Журавлев — статистическая обработка; Д. В. Плоткин, Т. И. Виноградова, Б. М. Ариэль, П. К. Яблонский — анализ полученных данных; Д. В. Плоткин, Б. М. Ариэль, М. В. Синицын, Е. М. Богородская — подготовка текста; Т. И. Виноградова, Ю. Р. Зюзя, П. К. Яблонский — редактирование.

Соблюдение этических стандартов: исследование одобрено этическим комитетом Санкт-Петербургского научно-исследовательского института фтизиопульмонологии (протокол № 73 от 23 декабря 2020 г.). Все манипуляции с животными проводили в соответствии с требованиями European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes (CETS No. 170), а также руководствуясь «Правилами работы с лабораторными грызунами и кроликами» (ГОСТ 33216-2014).

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Статья получена: 30.06.2021 **Статья принята к печати:** 21.07.2021 **Опубликована онлайн:** 04.07.2021
DOI: 10.24075/vrgmu.2021.036

Currently, it is believed that a quarter of the world's population is infected with tuberculosis; more than 10 million new cases of the disease and 1.2 million deaths from tuberculosis are registered annually. According to the World Health Organization, 15–20% of tuberculosis cases are extrapulmonary, and tuberculous peritonitis (TP) is the sixth most common of them [1, 2].

The concepts of pathogenesis of extrapulmonary tuberculosis were formed in the 30s–60s of the last century on the basis of clinical observations, experimental work and results of autopsies. TP was considered as part of polyserositis that simultaneously involved peritoneum, pericardium, pleura and synovial membranes of large joints in a specific process [3, 4] that was a typical manifestation of progressive primary tuberculosis.

The TP observed in the recent decades in the vast majority of patients develops during late stages of tuberculosis infection. Its pathogenesis is studied insufficiently; probably, TP is the result of lymphohematogenous spread of Mycobacterium tuberculosis (MBT) from the lungs, intra-abdominal lymph nodes, intestines, fallopian tubes [5, 6]. It is still unclear why, in such cases, generalization of the infection induces inflammatory process in certain organs and what are the conditions that promote the development of pathological changes in the given anatomical region.

Until recently, it was believed that such conditions were mainly shaped by microcirculatory disorders, when the initial focal lesions appeared only against the background of certain functional restructuring of the microvasculature: its increase in volume, slowed blood flow, close contact with tissues (semi-closed microcirculation system, with pores and fenestra in the walls of capillaries) [7].

It is known that each form of extrapulmonary tuberculosis has its own locus minoris resistentiae, where the initial focal lesions develop in a strictly regular sequence. In the abdominal cavity, such locus is the ileocecal zone, where the blood flow can grow weaker because of the slowed intestinal passage (ileostasis) and presence of a relatively large mass of lymphoid tissue in mesenteric lymph nodes and Peyer's patches, which get “swamped” when the blood flow is slow [3, 8].

Microvasculature disorders cannot but play a significant role in the development of TP, although, of course, they are not the only reasons behind the disease's pathogenesis. Poorly understood immune defense mechanisms also play a certain part here, as they do in any other pathological process. With regard to TP, it is the protective part played by the peritoneum MBT interact with, as well as local immunity [9].

These insufficiently studied mechanisms can be investigated under experimental conditions on models that reproduce human TP with all its characteristic features. There is reason to believe that with this approach, it will be possible to understand how and to what extent damage to the peritoneum and protective reactions therein "work" and balance out on the level of the body as a whole.

We searched for publications covering TP modeling methods in the PubMed database and found nothing in the current literature. Deepening the search, we discovered works published in the 19th century and first half of the 20th century [10, 11] that contained a few descriptions of unsuccessful attempts at intraperitoneal infection of laboratory animals with MBT culture for the purpose of experimental reproduction of TP.

Thus study aimed to investigate the pathogenesis of tuberculous peritonitis in a reproducible biological model.

METHODS

The study involved 18 male Sovetskaya Chinchilla rabbits weighing 2.60–3.25 kg, obtained from the Rappolovo laboratory animal nursery (Kurchatov Institute; Russia). The animals had no external manifestations of a disease; they were quarantined for two weeks in the certified vivarium of the St. Petersburg Scientific Research Institute of Phthisiopulmonology. The conditions were similar for all the animals, the food regimen standard, with access to water ad libitum.

The rabbits were divided into two groups, test and control. The division factored in age of the animals, i.e., all rabbits in a group were of the same age, obtained from the nursery at the same time.

We relied on the original technique we have developed to model TP: intraperitoneal inoculation of 6 ml of suspension of *M. tuberculosis* $H_{37}Rv$ standardized virulent test strain (10^6 mycobacterial cells in 6 ml). To infect control group and test group animals, we used saline or aluminum hydroxide gel MBT suspensions, respectively. *M. tuberculosis* $H_{37}Rv$ test strain (TBC #1/47, Institute of Hygiene and Epidemiology; Prague, 1976), taken from the collection of the Scientific Centre for Expert Evaluation of Medicinal Products, was cultured on the Lowenstein–Jensen medium (Becton, Dickinson and Company; USA). The mycobacterial suspension of the three-week (second generation) *M. tuberculosis* $H_{37}Rv$ test strain was prepared *ex tempore* on the day of infection.

The animals were infected intraperitoneally with immunodeficiency in the background. To induce immunodeficiency, we decreased the concentration of tumor necrosis factor TNFα (the main cytokine, regulator of the formation and resistance of granulomas) by intravenous administration of the TNFα inhibitor infliximab (chimeric mouse-human monoclonal antibody) (BIOCAD; Russia). We also suppressed the phagocytic activity of peritoneal agranulocytes (peritoneal macrophages) through intraperitoneal administration of iron (III) hydroxide sucrose complex (PHARMASINTEZ; Russia). This drug was chosen as a donor of ferric iron to create the effect of "iron overload" in immunocompetent cells that absorb excess non-transferrin bound iron [12, 13].

The test group consisted of 10 rabbits, who were injected infliximab (once, into the marginal ear vein, 16 mg/kg) [14] one day before intraperitoneal infection with MBT culture, and 5 ml of iron (III) hydroxide of the sucrose complex intraperitoneally 1 hour before infection.

Eight rabbits of the control group were infected in a similar way, but they did not receive the TNFα inhibitor and the iron sucrose complex.

After infection, we monitored body weight of the animals (weighed them once every 10 days), observed their behavior, applied intradermal recombinant tuberculosis antigen (Diaskintest®) tests to confirm the development of tuberculosis infection. The animals were removed from the experiment on the 45th day from the moment of infection with the help of a general anesthetic overdose: sodium thiopental 250 mg and pipcuronium bromide 1 mg intravenously.

During autopsy, we looked for effusion in serous cavities and tubercles on the serous integuments of the liver, spleen, intestines and lungs, and assessed the shape and size of the intrathoracic and intra-abdominal lymph nodes, the size and structure of the kidneys. For microscopic examination, we sampled tissues of the parietal peritoneum, liver, kidneys, spleen, lungs, heart and lymph nodes. The samples were embedded in paraffin wax. Slices were stained with hematoxylin and eosin; we also applied the Van Gieson and Perls staining methods to identify collagen fibers and have a qualitative reaction to ferric iron, respectively. Besides, to detect MBT, we stained the samples following the Ziehl–Neelsen technique and then performed a bacterioscopic study. Also with the aim

of detecting MBT DNA, we subject peritoneal and omentum tissues to PCR test using the Amplitub-RV reagent kit (Syntol; Russia).

RESULTS

Dynamic post-infection observation of the animals did not reveal any weight loss. On the contrary, all rabbits gained an average of 132 ± 42.6 g over the entire period of the experiment. On the 20th — 22nd day after the infection, the recombinant tuberculosis allergen (Diaskintest®) test returned hyperergic in all 18 rabbits.

Autopsy and histological examination of the samples revealed significant differences between the rabbits of test and control groups.

On the 45th day from the moment of intraperitoneal infection, serous cover of the abdominal cavity of control group animals remained intact at both macro- and microscopic levels. There were no specific changes identified either in the parenchymal organs of the abdominal cavity or in the kidneys. PCR test did not detect MBT DNA in the animals' peritoneum and omentum.

At the same time, 3 animals (37.5%) had clear signs of tuberculous pneumonia. Lung tissue microscopy revealed BLURRY, fused macrophage granulomas with small areas of necrosis and mild leukocytic infiltration along the periphery, and occasional larger foci of caseous necrosis infiltrated by decaying leukocytes. Distelectasae dominated by atelectasae, focal exudative reaction, vasculitis products were found in the adjacent lung tissue (Fig. 1A, C). In such areas, the pleura was thickened due to edema and mononuclear infiltration. Van Gieson staining revealed no encapsulation of foci of caseous necrosis, nor signs of organization in the areas of tuberculous inflammation. Ziehl–Neelsen staining allowed detecting microcolonies of MBT in the necrosis foci (Fig. 1B).

It is worth noting mild character of reactive changes in individual lymph nodes of the abdominal cavity and mediastinum and lack of signs of specific tuberculous lymphadenitis. Microscopic examination revealed focal nonspecific infiltration of the peritoneum with MBT, where bacterioscopy failed to detect the bacteria. Their absence in such areas of the peritoneal cover was confirmed by the PCR test.

In the test group, in the first week after infection all animals were showing clinical symptoms of intoxication: physical inactivity, lack of appetite, loss of body weight by 75–110 g (average — 84 g). In the next 35 days, the dynamics of their state was positive, with physical activity, appetite returning to norm and some body weight gained.

Forty–five days after infection, autopsy of all test group animals presented characteristic macroscopic signs of TP: yellowish serous effusions in the abdominal cavity (maximum volume — 5 ml), dense and loose adhesions of varying degrees of maturity between the small intestine loops, the greater omentum and the anterior abdominal wall, multiple small and large tubercles measuring 2–6 mm on the parietal and visceral peritoneum (Fig. 2). Histological examination of the adhesions and serous cover fragments revealed macrophage granulomas with single giant multinucleated Langhans cells, as well as extensive foci of caseous necrosis with perifocal accumulation of macrophage granulomas merging with each other (Fig. 3A, B). Similar changes were also present in the mesentery of the small intestine and the greater omentum.

Nine out of ten (90%) test group animals had active pulmonary tuberculosis in the form of foci of caseous necrosis with small accumulations of lymphocytes along the periphery and specific granulations. Van Gieson staining revealed no signs of organization in the tuberculous inflammation foci in the lungs. Ziehl-Neelsen staining allowed discovering MBT microcolonies in caseous masses.

Perls staining uncovered multiple accumulations of macrophages with a high content of iron in the cytoplasm. Such were found both in the tissues of the peritoneum and those of the lungs (Fig. 3C, D). One case (10%) was diagnosed as tuberculous splenitis, another (10%) as tuberculous hepatitis. In the lymph nodes of the small intestine mesentery both specific changes and nonspecific reactive hyperplasia of lymphocytes were revealed. At the same time, all rabbits had dystrophic changes in the kidney convoluted tubes epithelium, liver and myocardium, which were accompanied by pronounced plethora and edema of the organ stroma.

Changes in the serous membrane of the intestine were of two kinds: in some cases, productive tuberculous peritonitis was detected without signs of organization, and in others it was nonspecific reactive inflammation. No specific inflammatory changes were found in the serous membrane of the stomach. Tests for MBT DNA in the tissues of the greater omentum returned positive in all 10 cases.

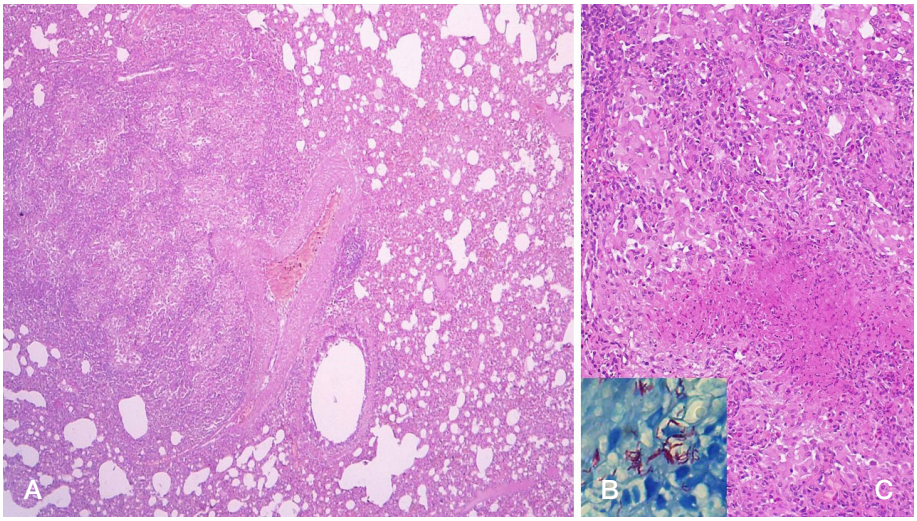

Fig. 1. Foci of tuberculous lesions in the lung of a rabbit. **A**. Lung area under tuberculous granulomatous inflammation with distelectasis (micropreparation, stained with hematoxylin and eosin; ×40). **B**. Acid-resistant mycobacteria in the focus of tuberculous inflammation (micropreparation, staining according to Ziehl–Neelsen; ×1000). **C**. Lung: a focus of caseous necrosis with leukocyte infiltration and perifocal macrophage-epithelioid granulomas (micropreparation, stained with hematoxylin and eosin; ×100)

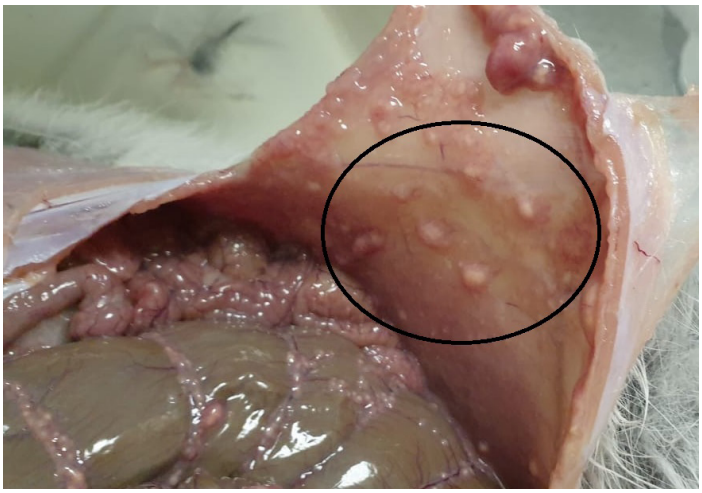

Fig. 2. There are multiple tuberculous tubercles on the parietal peritoneum of a rabbit-disseminates (circled in a *round frame*) (macro specimen)

DISCUSSION

The choice of rabbit (Oryctolagus cuniculus domesticus) as a laboratory animal in the development of the experimental TP model was a thought-out decision. According to Claude Bernard, a good choice of animal is often enough to solve the most difficult general questions.

Previous research has shown that rabbits, which are considered resistant to M. *tuberculosis*, under certain conditions of can naturally develop specific chronically progressive granulomatous inflammation or latent tuberculosis infection, depending on the MBT virulence [15–17]. From the morphological point of view, the subject is the evolution of granulomas — their formation, maturation and decay. In rabbits, these processes run the same way as they do in human being. Rabbits are fairly large laboratory animals, so selecting them as experimental subjects researcher can reliably monitor pathophysiological changes from the very beginning of the infectious process until its completion, even without resorting to euthanasia [18].

According to our observations, in case of intraperitoneal infection with MBT rabbits with an intact immune status develop not TP but tuberculous inflammation of the lungs and mediastinum lymph nodes. Only half of the infected animals fall ill; the changes in their lungs and lymph nodes are transient and disappear 1.5 months after infection. As inflammatory changes disappear from the organs, so do the MBT. The absence of inflammatory changes in the abdominal cavity is due to the serous membrane playing the part of a mechanical barrier in the pathological process, as does, for example, the greater omentum, which limits inflammatory foci in the abdominal cavity independently and as a sui generis organ of the humoral and cellular defense system.

It is now generally accepted that there are three cellular systems opposing the invasion of microorganisms in the abdominal cavity: lymphocytes, mesotheliocytes, and peritoneal macrophages. They are the first line of defense, which relies on phagocytic activity and production of inflammatory cytokines [19]. Secretory function of the mesothelium also contributes to the body's defensive capabilities, both under normal and pathological conditions [20]. It makes the MBT release into the free abdominal cavity, where they die in the exudate containing free antibodies, peritoneal macrophages and neutrophilic leukocytes, which, taken together, produce bacteriostatic and bactericidal effects. In other words, we consider the peritoneum not as a morphologically formed statically permanent formation but as an organ of humoral and cellular immunity [21].

Obviously, physiological activity of the peritoneal macrophages is important for the peritoneum's protective properties. Having suppressed it with infliximab (TNFα

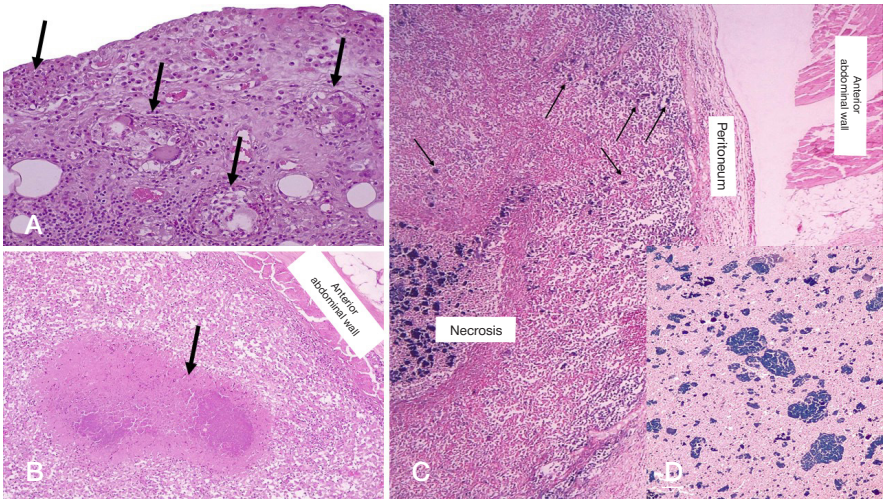

Fig. 3. Tuberculous peritonitis in rabbits. Micropreparation. **A**. Tuberculous granulomatous peritonitis (granulomas are shown by arrows) (staining with hematoxylin and eosin; ×200). **B**. Caseous peritonitis (a focus of caseous necrosis is shown by the arrow) (stained with hematoxylin and eosin; ×100). **C**. Accumulations of iron-containing pigment in necrotic masses and in adjacent macrophages (iron-containing pigment is blue-green, some of the macrophages with pigments are shown by arrows) (Perls reaction; ×100). **D**. Fragment of Fig. C (Perls reaction; ×200)

inhibitor) and overloaded with iron, we were able to reproduce chronically progressive TP with the formation of alterative and granulomatous changes against the background of MBT reproduction. Speaking about the importance of protective reactions in the pathogenesis of TP, we mean not only phagocytosis of pathogens by macrophages but also the further fate of both. MBT partially or completely die in the macrophages' cytoplasm, while the affected cells themselves are removed by the body naturally: secreted into the free abdominal cavity from the peritoneum and cleared and from the lungs.

Thus, in addition to presence of an etiological factor in the abdominal cavity TP development requires inactivation of peritoneal macrophages and regulatory cytokines, the main of which is TNFα. The latter plays an important part in tuberculosis protection in human beings, too. It performs many immunoregulatory functions, including early induction of chemokines that leads to the recruitment of leukocytes, and it also participates in the morphogenesis of granulomas, which always appear in people suffering from a mycobacterial disease [22, 23].

Genetic studies have shown that toll-like receptors, such as TLR-2, significantly affect the susceptibility (consequently, resistance) to tuberculosis. Variants of their polymorphism were examined mainly in pulmonary tuberculosis; however, a significant relationship was found between the TLR-2 Arg753Gln polymorphism and the incidence of TP. This suggests that individuals with a TLR2 gene Arg753Gln polymorphism run an increased risk of TP [24].

Our experimental TP modeling allowed discovering and number of important features of its pathogenesis.

1. With a TNFα inhibitor and an iron (III) hydroxide sucrose complex administered, all animals naturally develop a widespread, actively progressive tuberculous inflammation of the peritoneum dominated by granulomatous-necrotic changes that sometimes turn into caseosis. Its expansive character is proven by the fact that, in addition to the parietal peritoneum and the greater omentum, tuberculous inflammation spreads into the visceral peritoneum of the mesentery and intestinal loops.

2. With the described method for TP modeling there also develops a destructive pulmonary tuberculosis with lymphadenopathy of intrathoracic and intra-abdominal lymph nodes where specific granulomatous and nonspecific hyperplastic changes can be found.

3. As for the other organ lesions of hematogenous genesis (those of liver, spleen, kidneys), this method of modeling only allowed finding them in isolated cases as evidence of hematogenous generalization of the infection.

Our experimental TP model has reliably reproduced the clinical, morphological and pathogenetic features of tuberculous lesions of the peritoneum of human beings. They can be observed in case of hematogenous dissemination of pulmonary tuberculosis, which develops against a particular comorbid background that has the peritoneum's local immunity compromised and/or general immunosuppression developed.

As we learned from the literature and our own clinical observations, peritoneal tuberculosis develops during pregnancy, in patients with diabetes mellitus, liver cirrhosis, cardiovascular diseases comorbid with ascites, as well as peritoneal dialysis, congenital and acquired immunodeficiencies.

Immunosuppression is common in pregnancy. It is associated with a complex system of immunological interaction between the mother and the fetus. Progesterone and cortisol, the levels of which go up during pregnancy, suppress T- and

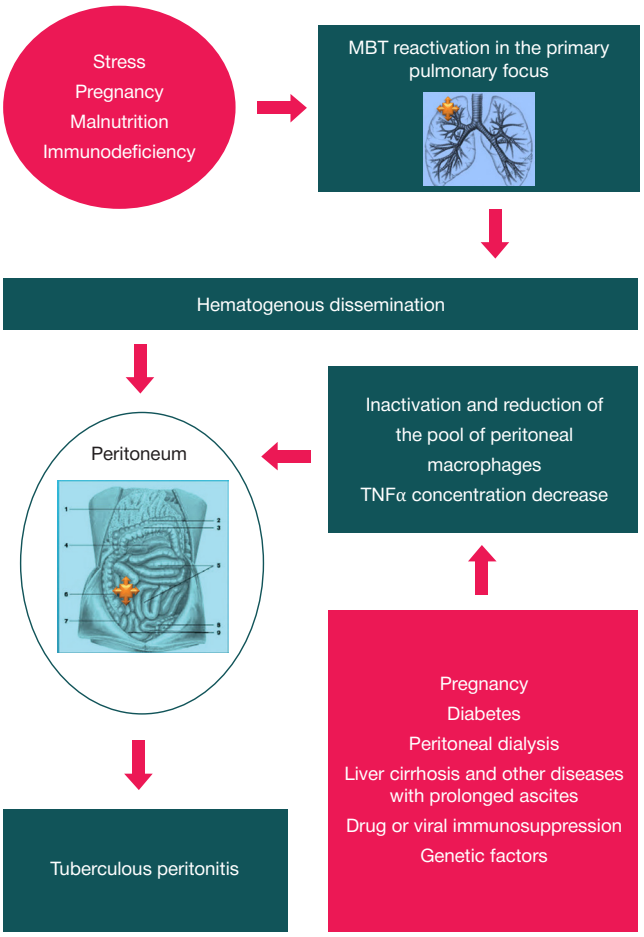

Fig. 4. Tuberculous peritonitis pathogenesis pattern

B-cell immunity, and after 2–3 months after childbirth, the pool of agranulocytes is depleted [25]. It affects the number and activity of peritoneal macrophages, as indicated by the incidence of TP in the first months after birth.

Both during the carbohydrate metabolism decompensation stage and when the compensation is achieved, diabetes mellitus patients have the induced production of IL1 and TNFα by peripheral blood mononuclear cells (compared with healthy individuals) going down and spontaneous — going up [26], which leads to disruption of the morphogenesis of granulomas and reduces their resistance. This explains the cases of detection of TP in such patients.

With the above pathology in the background, persisting ascites, as well as with carcinomatosis, lead to chronic aseptic inflammation of the peritoneum, the outcome of which significantly decreases the number of macrophages, including peritoneal ones. At the same time, the local concentration of TNFα decreases [27].

Chronic peritoneal dialysis is also directly associated with the development of peritoneal tuberculosis. Dialysis solutions contain an increased concentration of glucose and have a non-physiological pH value, which entails disturbance of the functional activity of phagocytes and lymphocytes in the peritoneum [28].

Immunodeficiency states, both congenital and acquired, including after the use of immunomodulatory drugs (TNFα inhibitors) and glucocorticosteroids, entail a decrease in the number of agranulocytes and TNFα synthesis. Low concentrations of TNFα and inactivation of macrophages in the peritoneum directly contribute to the development of TP. And, of course, HIV infection with its characteristic

immunosuppression can become a trigger in the pathogenesis of peritoneal tuberculosis [29, 30].

Analyzing the results of our experiment and taking into account clinical observations and literature data, we can propose the following pattern of TB pathogenesis in humans. Stressful influences, such as malnutrition, pregnancy, a sudden change in social status, immunosuppressive diseases etc., lead to the reactivation of the primary tuberculous focus and destruction of previously formed granulomas and lymphohematogenous by the spread of MBT [31]. Where, in which anatomical region the secondary focus of extrapulmonary tuberculosis infection will develop directly depends not only on the characteristics of microcirculation in this region but also on the local immune protection (local immunity according to A. M. Bezredka). For the peritoneum, a smaller pool of peritoneal macrophages and weaker cytokine production is a necessary and sufficient condition to have TP developing therein. Figure 4 shows the pattern of TP pathogenesis.

CONCLUSIONS

Clinical manifestations, methods of detection, diagnosis and treatment of respiratory tuberculosis are well studied and fully covered in the scientific and medical literature. Tuberculosis mainly affects the pulmonary parenchyma, but under certain conditions associated with changes in local and humoral immunity, the process can localize in almost all organs and tissues of a human being. As a rule, actively progressive pulmonary tuberculosis receives the bulk of attention while much less effort goes into investigating extrapulmonary manifestations of the infection with severe destructive changes. Over the past decades, about 15% of the detected tuberculosis cases worldwide are extrapulmonary. Their clinical diagnosis is fraught with objective difficulties, since in extrapulmonary tuberculosis, the results of the gold diagnostics standard — testing sputum for MBT — often return negative. And, in the most paradoxical way, the presence of *M. tuberculosis* in extrapulmonary foci often causes a wide range of nonspecific symptoms, thereby creating additional diagnostic difficulties. TP is one of the extrapulmonary forms of tuberculosis that are difficult to diagnose and treat. In this work we have experimentally shown the role of peritoneal macrophages in the formation of local protective immunity and TNFα in the humoral aspects of the protective processes. The data obtained will make the features of the modern pathogenesis of tuberculous peritonitis more clear and enable development of the diagnostic criteria and therapeutic (including surgical) protocols for such patients.

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