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Expression of Somatic Multiple Drug Resistance Genes, mdr1a and mdr1b, is Increased with the Progression of Tuberculous Inflammation in Mice [Version 1, 2 Approved with Reservations]

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Abstract

Somatic multiple drug resistance (MDR) is a poorly understood phenomenon which was primarily manifested in the treatment of cancer. It is characterized by a reduced susceptibility of tumor cells to antitumor drugs due to a release the drugs out of the cells. So called drug resistance proteins are responsible for this phenomenon that act like pumps. Prolonged use of drugs leads to enhanced expression of the genes encoding these proteins and their increased activity. Such cellular resistance makes sense in survival of somatic cells when exposed to different harmful substances. Here we have shown that tuberculosis infection itself causes increased expression of mdr1a and mdr1b MDR proteins that along with the possible development of cellular resistance to anti-TB drugs complicates the treatment of tuberculosis.

The problem of drug resistance is one of the challenges facing modern Phthisiology [1]. The formation and activation of the drug resistance for antibacterial therapy can occur at two levels of organization: at the level of microorganism (*M. tuberculosis*, MBT) and host cells (cells of the lung). The mechanisms and causes of resistance development of MBT to anti-TB drugs are actively studied, while the analysis of the activation mechanisms of drug resistance in somatic cells to these drugs is only in the initial stage [2,3].

Problem of somatic drug resistance first emerged in connection with the failure of chemotherapy of cancer patients. At present, scientific direction is formed which has convincingly shown that the drug impact on tumor cells triggered mechanisms leading to the development of drug resistance (including multiple, MDR) in tumor cells of different histogenesis [4,5].

First, the mechanisms of MDR are implemented by special transport proteins, localized predominantly on the plasmatic membrane and acting as "pumps". These pumps use for their activity the ATP energy: the result of the work of these proteins is decrease of intracellular accumulation of drugs through their removal from cytoplasm into extracellular environment [6-8]. Such proteins are called as "MDR proteins of somatic cells" and P-glycoprotein (Pgp) first opened is the most universal of all MDR proteins [9,10]. High expression of Pgp is characteristic for the "barrier" cells such as epithelium and endothelium [11], a moderate presence of Pgp has been observed in macrophages [11]. In addition to Pgp other proteins responsible for the development of MDR of somatic cells: MRP1, BCRP and LRP were identified in the lungs [12]. Each of these proteins is characterized by its structural features, localization and "preferences" in the binding of certain chemical components, which include pharmaceutical compounds of different nature [13-16]. It is assumed that in the norm these proteins involve in limitation of penetration of various toxins from air through the broncho-alveolar epithelium [17,18].

The role of these genes in pathophysiology of respiratory diseases and their treatment is not always possible to clearly identify that is associated with the diverse cellular composition and complex organization of lung tissue. In the conditions of development of pathological process and on the background of prolonged chemotherapy exposure, the protective function of MDR proteins may also expand due to changes in their functional activity, affinity to drugs, and execution of non-canonical functions. From this point of view, the functional activity of the Pgp protein (MDR1) is the most relevant. In recent years there is evidence showing that Pgp is a multifunctional protein and may take different forms: in the role of the cellular "pump" as well as in the role of a regulator of various cellular processes [19]. A number of studies have shown that not only inflammatory factors affect the expression of Pgp, but the protein itself has a regulatory influence on the development and severity of inflammatory response [20-22]. Currently, the increased activity Pgp (MDR1) is regarded as one of the most important factors in the survival of the cells of the body in the adverse conditions of the pathological process and toxic effects. In this regard, the aim of this study was to determine the peculiarities of expression of mdr1a and mdr1b genes in the cells of lung tissue of mice with the progression of tuberculous inflammation.

Material and Methods

M. tuberculosis strain H37Rv was originally obtained from the Institute Pasteur, Paris, France (a kind gift of G. Marchal). To increase the virulence, culture was passaged through mice twice. The final culture was washed in phosphate-buffered saline (PBS) with 0.05% Tween 80, resuspended in PBS with 0.01% BSA and 0.05% Tween 80, dispensed in aliquots into polypropylene vials, and frozen at -80°C. CFU of the frozen aliquots were determined after thawing by plating serial 10-fold dilutions on 7H10 agar.

Male BALB/c inbred mice (22-23 g of body weight) were used. Mice were bred under conventional conditions at the Animal Facilities of the Central Institute for Tuberculosis (CIT, Moscow, Russia), in accordance with the guidelines from the Russian Ministry of Health # 755, NIH Office of Laboratory Animal Welfare (OLAW) Assurance #A5502. Water and food were provided ad libitum. All experimental procedures were approved by the CIT animal care committee (IACUC protocols #4). Mice were infected by the respiratory route with 100 viable CFU/lung using an inhalation exposure system (Glas-Col, Terre Haute, IN). All animals were divided into 2 groups: control animals (n=10) and infected (n=30). The animals were removed from the experiment after 21, 45 and 90 days after infection.

Histological Preparations

To assess the stage of tuberculous process a standard histological preparation of lung of mice was used. Staining with hematoxylin and eosin was performed according to standard methods. Photography was made on light microscope Leica DM1000 LED(Germany) using the lens N PLAN 100x/1.25 Oil and camera (Leica).

RT-PCR

For PCR with reverse transcription (RT-PCR) of MDR protein gene every tissue sample was homogenized in 1 ml Tri-Reagent (MRC Inc., USA). Total RNA from the lung of individual mice was isolated in accordance with the Protocol of the manufacturer Tri Reagent. RNA was diluted with deionized water to a final concentration of 0.5-1 μg/μl. The quality of the selected RNA was checked by electrophoresis in 1% agarose gel. Samples with clearly visible bands of 18S and 28S RNA was used for further analysis. Gel was photographed with the help of video "DNA Analyzer". RT-PCR was performed using Oligo(dT)16 primers ("thermo Scientific"). The composition of the reaction mixture for the reaction of reverse transcription: a buffer for reverse transcription, a mixture of deoxynucleotide-triphosphates (dNTP) 2.5 mM each, Oligo(dT)$_{16}$ primers - 0.5 μg per

sample, RNAse inhibitor (Ribolock RNase "thermo Scientific") 20 units per sample, reverse transcriptase (RevertAid Reverse "thermo Scientific") – 100 units, total RNA 2 мкг Next, the vials with reaction mixture were placed in a programmable thermostat and incubated for 50 minutes at 42 °C. To equalize the amount of cDNA in different samples household gene β-actin gene was amplified. For amplification of cDNA specific primers (SINTOL) were used:

Table 1: Primers for amplification.

Gene	Primer	Size of product
mdr1a	sense: 5'- GACGGACAGGACATCAGAACC -3' antisense 5'-TTCAACTTCAGGATCCGCCA	752**п.н**.
mdr1b	sense: 5'-GACGGACAAGACATCAGAACC-3' Antisense: 5'-GCAAACACTGGTTGTATGCAC-3'	823 **п.н**.
b-actin	Sense: 5'-CAGCTTCTTTGCAGCTCCTTC-3' Antisense: 5'-GTACATGGCTGGGGTGTTGA-3'	451п.н.

PCR was performed on thermocycler "Tertsik" ("DNA technology", Russia). Conditions of amplification: 94°C 30 s, then 25 to 40 cycles at 94°C, 10 s, 60°C 10 s, 72°C 10 s, and then 72°C 1 min Final concentration of the enzyme (Tag DNA Polymerase "thermo Scientific") for the polymerization of – 1 units on trial. The number of cycles were selected in such way as to be in the exponential phase of formation of the reaction products. Amplification products (20 µl reaction mixture) were separated by electrophoresis in 2% agarose gel with ethidium bromide. Gel was photographed with the help of video "DNA Analyzer".

The level of MDR of gene expression was assessed by luminescence intensity of amplification products in the gel. For semi-quantitative analysis of the gel the photographs were processed using the software Image-ProPlus 6.0.0.260 (Media Cybernetics, Inc). The luminescence intensity strand of DNA was determined according to brightness of the respective pixels in the digital image in values range from 0 to 255 (8-bit coding system of colors). Normalization was performed by gene β-actin (house-keeping gene). For each sample, the brightness of the strips of β-actin was taken as 100. For products of amplification of other genes, the brightness values were calculated on this level. To assess the differences between samples used non-parametric U-Mann Whitney for small samples.

Results and Discussion

The Change in Lung Tissue in Dynamics of Progression of Experimental Tuberculosis

At aerosol inoculation with *M. tuberculosis* mice develop exudative-necrotic inflammatory process the severity of which varies in different parenchymatous organs and depends on the model of infection. Our model allows to perform study of parenchymatous organs within a few months and identify the relationship between the level of expression of MDR genes with the stage of tuberculous inflammation. Lungs of control uninfected animals have a characteristic "air" structure of tissue (Figure 1a). Twenty-one days after Mtb inoculation, characteristic changes in the lungs, indicating the presence of specific inflammation were observed. In pulmonary parenchyma there are some small perivascular infiltrates (pneumonic foci, PF), consisting mainly of mononuclear cells. The rest of the parenchyma retains airiness. Inter-alveolar partitions are in places thickened due to interstitial edema, expansion of sections of the capillary network (Figure 1b). Forty-five days after infection the pneumonic foci were determined containing clusters of foamy macrophages, among of which there are single cells with completely destroyed cytoplasm. Here small clusters of polymorpho-nuclear leukocytes (PNL) were determined. Inter-alveolar partitions are markedly thickened due to infiltration with mononuclear cells mixed with PNL that also was determined in the Lumina of the capillaries. Increased permeability of the vascular network was demonstrated by pronounced diapedesis of red blood cells, the presence of cellular elements of inflammation in intra-alveolar space (Figure 1c). 90 days after infection large perivascular infiltrates were determined in the lungs of mice, which merge together to form a large PF. They consist a lot of foamy macrophages, some are at the stage of destruction. Around of these cells PNL are concentrated.

The area of the air parenchyma reduced to 40-45% of the area of the histological slice. Inter-alveolar partitions throughout are thickened due to edema, infiltration with celle elements of inflammation. Blood network is full-fledged, there are hemorrhages (Figure 1d).

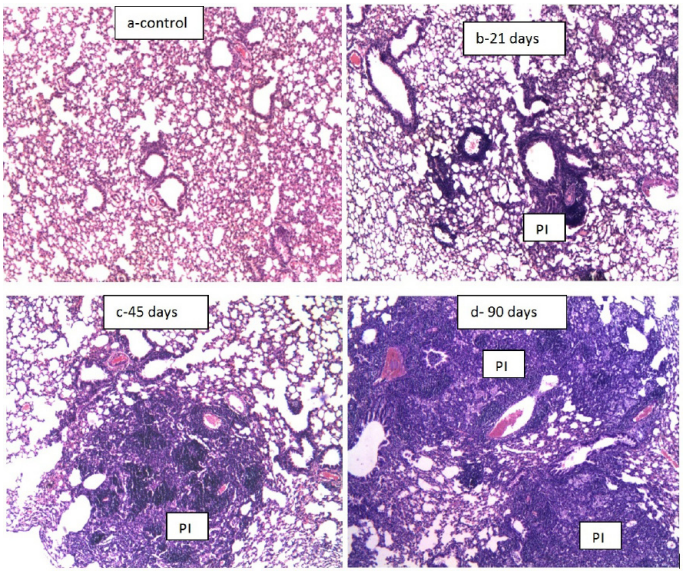

Figure 1: Dynamics lung pathology in mice infected with *M. tuberculosis*. PI - perivascular infiltrates.

Determination of MDR Gene Expression in Lung Cells at Progression of TB Infection

Data on expression of mdr1a and mdr1b genes at different stages of TB severity and tissue destruction are shown in Table 2 and in Figure 2. The mdr1a gene expression linearly increases with duration of TB process, reaching a maximum at 90 days. The expression level of this gene was 3.5 fold higher as compared to the control group (p=0.02). The expression of mdr1b gene was significantly increased at 45 days (p=0.03), almost 2-fold higher in comparison to control group and remained at high level during further development of tuberculous inflammation.

Table 2: Level of MDR gene expression at different stages of TB infection.

	mdr1a	mdr1b
Control	33* 21-48**	39 28-44
21 days	94 54-102	35 22-52
45 days	89 43-128	79 74-100
90 days	118 76-141	61 48-84

*mediana, **25-75% distribution

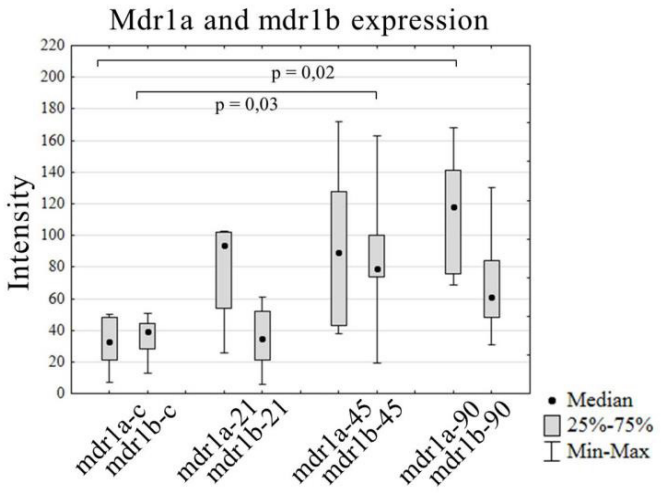

Figure 2: Alteration in the expression of mdr1a and mdr1b in the progression of tuberculous inflammation.

Thus, it is possible to note that expression of mdr1a and mdr1b genes increases when TB process develops and severity of inflammation escalates. The data obtained allow to conclude that pathophysiological factors of tuberculous inflammation act themselves as inducer of the expression of these genes.

So, we can talk about important role of MDR genes, in particular Pgh, in respiratory diseases. Pgp (170kDa) mainly localized on the apical surface of the plasma membrane of alveolocytes. A small amount of the protein was found in the endoplasmic reticulum and the Golgi apparatus. It is noted that the release of Pgp substrate - rhodamine123 - comes predominantly from the basolateral surface to the apical cell [18]. However, the absence of functionally active Pgp on the apical surface of the plasma membrane leads to disruption of the processes of the directed transport [17].

The clinical significance of high level expression of MDR proteins was demonstrated in chemotherapy of different types of human cancers. For example, at different variants of lung cancer the relationship between the increase in the level of expression of MDR proteins resulted from the effects of anticancer drugs and decrease the effectiveness of chemotherapy was demonstrated. It was also shown that the increase of level of MRP1, LRP, and MDR1 expression correlated with the development of invasive potential of the tumor, the risk of metastases and relapse, i.e., high expression of MDR proteins leads to an unfavorable prognosis of the disease [23].

Various cytokines, interleukins 2 and 4, tumor necrosis factor (TNFa) also increase the expression and activity of MDR1/Pgp [21,22]. In turn, mice knock-outed on mrp1 and mdr1a/1b genes demonstrated a reduced inflammatory response in the lungs during its induction by different factors as compared to wild type mice [20]. It is assumed that not only inflammatory process affects on expression of MDR proteins, but these proteins themselves influence on development and severity of inflammatory response. At development of pathological process the Pgp possesses multifunctionality which is associated with the inhibition of apoptosis by suppressing the expression of endogenous TRAIL protein, and subsequent decrease of activity of caspase 8 and 3 (TRAIL ligand, belonging to the family of TNF) [19].

Previously, in model system of in vitro, we have demonstrated the possibility of somatic cells of different origin - monocyte-macrophage and epithelial cells to form drug resistance (survival at toxic concentrations of the drug) to rifampicin and nano-rifampicin. It as shown that such resistance of somatic cells to rifampicin is formed in presence of sub-toxic drug concentration: IC_{50} in the first 3 months increased by 1.5-2.5 times [24]. Cells resistant to rifampin are characterized by high functional activity of one of the key protein MDR – PGP [24].

It should be noted that preparations of antiretroviral therapy against human immunodeficiency virus (HIV) are also substrates for Pgp [22]. In this context, the increased expression

and activity of MDR1/Pgp in patients with tuberculosis combined with HIV can be one of the reasons for reduced effectiveness of chemotherapy.

Currently Pgp assumed to be a regulator of cellular processes, providing a better cell survival in adverse conditions. It is discussed that Pgp can take different forms – as a cellular "pump" and in the role of a regulator of protective cellular processes. This proposition may be important in the light of the strategy against tuberculosis.

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