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Level of cytokines in patients with pulmonary drug susceptible and resistant tuberculosis

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Abstract

Background: Cytokines are the regulators of the immune response in tuberculosis: TNF-alpha and CXCL8 (IL-8) are involved in the granuloma formation, IL-10 inhibits the inflammation; some chemokines increase the liver production of the acute phase proteins (APPs). The aim of the research was to assess the serum level of IL-8, TNF-alpha, IL-10, C-reactive protein (CRP), ceruloplasmin and fibrinogen in patients with drug-sensitive and multidrug resistant tuberculosis (MDR-TB).

Material and methods: A prospective case-control study, which included 51 patients, distributed in 2 groups: the 1st study group (N=24 new cases with drug-sensitive TB) and the 2nd study group (N=27 new cases with MDR-TB) according to sex and age were compared with the control group (N=36 healthy individuals).

Results: Serum concentration of IL-8 was elevated up to 13 times, TNF-alpha up to 4 times and IL-10 up to 2 times in study groups, compared with the reference value of the control group. Fibrinogen concentration was elevated up to 2 times in study groups compared with the control group and CRP up to 3 times compared with conventional value. Ceruloplasmin was statistically higher in the drug-sensitive TB and mildly elevated in MDR-TB group.

Conclusions: Proinflammatory biomarkers are more elevated than the anti-inflammatory response, without differences among groups regarding drug sensitiveness.

Key words: tuberculosis, immunity, biomarkers.

Introduction

Evolution of tuberculosis is conditioned by the Mycobacteria tuberculosis (Mtb) virulence, the organism's protective mechanisms and capacity to maintain the infection in latent state [1]. Mtb is an intracellular pathogen with a high capacity to escape from the immune host defenses. About 1,7 billion people, 23% of the world's population are infected with Mtb, but only 10% of infected individuals will develop active tuberculosis, more frequently pulmonary forms [2]. The delay between the infection and the evolution towards an active disease differs due to the complexity of the immune suppressive risk factors [3]. In the first two years after the infection 5% of infected individuals will fall ill and 5% in a later period of life. People co-infected with HIV have a 5-15% per year risk to fall ill with tuberculosis [4].

The most important innate immune cells involved in the Mtb infection are: macrophages, dendritic cells and natural killer cells [5, 6, 7, 8]. These immune cells express a range of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptors [9]. The activation of PRRs will stimulate the host defense functions: phagocytosis, autophagy, apoptosis and inflammatory cascade activation [9, 10]. Following the entrance in the pulmonary alveolus the Mtb infects the 2nd type pneumocytes and polymorphonuclear neutrophils. After the death of the infected cells, Mtb is phagocyted by the alveolar macrophages, which are the first immune cells involved in the innate immunity and in the recruitment of the monocyte-derived macrophages during the early stage of the tuberculous infection [11]. Among the innate immune cells, the macrophages play the major role in the host resistance against Mtb through multiple ways: production of the oxygen free radicals, nitric oxides, cytokines, phagosome acidification and autophagy of the infected cells [12, 13, 6, 14].

Before the onset of the proinflammatory cytokine cascade, the innate immune cells recognize Mtb through the pathogen-associated molecular patterns (PAMPs). The PAMPs molecules are glycolipids, lipoproteins and carbohydrates, which are encountered in the Mtb walls [9]. The recognition of the PAMPs by the PRRs of the alveolar macrophages induces the production of the proinflammatory cytokines, more expressed IFN-gamma, IL-12, IL-1β and macrophage inflammatory protein-1α [15, 6, 16]. There are two types of macrophages involved in the innate resistance: classically activated macrophages (CAM) – M1 and alternatively activated macrophages (AAM) – M2. The CAMs are induced by the interleukins secreted by the T lymphocytes

and have a high bactericidal activity. The alternatively activated macrophages (AAMs) have a reduced antigen processing capacity and are induced by the cytokines produced by the T helper 2 lymphocytes: IL-4, IL-13, IL-10 and TGF-β through the ligation with PAMPs and early secretory antigen 6 (ESAT-6) derived from Mtb [15, 17, 6]. The role of AAMs is to maintain the balance between the active mycobacterial growth and disease evolution by suppressing the T helper 1 lymphocytes [18]. Foam cells are the fat-laden alternatively activated macrophages identified in the tuberculous granuloma and their formation is triggered by the uptake of the modified low density lipoproteins into the monocyte-derived cells. Recently another population of macrophages involved in granuloma was described and defined as termed myeloid suppressor cells (MSCs). MSCs suppress T-cells responses through the secretion of the anti-inflammatory cytokine IL-10 and transforming growth factors [17]. Other innate-like cells involved in the immune defense against tuberculosis are: mucosal associated T cells, CD-1 restricted lymphocytes and natural killer T cells. It was established the role of the airway epithelial cells and mast cells in the early immune response against Mtb invasion [19].

The dendritic cells are important innate immune cells responsible for the recruitment of different cells to the site of infection due to their capacity to present the antigens to T lymphocytes from the lymph nodes, where the cell mediated immune response is primarily developed. During the primary infection, the ligation of the dendritic cell receptors by mannose-capped lipoarabinomannan of the Mtb reduces the production of the anti-inflammatory cytokine IL-10, which diminishes their maturation and decreases the production of other stimulator cytokines. The polymorphonuclear neutrophils are also involved in the innate immunity by the production of the free oxygen radicals, initiation of the inflammatory process and constitution of the granuloma, through the secretion of chemokines IL-8 and MCP-1 [20, 16, 19, 21].

The hallmark of the Mtb infection is the granuloma formation. It is a histopathological structure developed by the host to contain the infection in the latent state and to eliminate the mycobacteria. Tuberculous granuloma is composed by a large spectrum of modified macrophages: multinucleated giant cells (Langhans giant cells), epitheliod cells and foam cells, surrounded by a crown of T lymphocytes. In the centre of the granuloma is placed a necrotic region with a characteristic caseation appearance. It was recognized that the cell apoptosis is bactericidal against the Mtb and promotes the antigen presentation, but the caseous necrosis releases the Mtb and contributes to progression of the inflammation and tissue damage [7, 8, 22].

The recognition of the PAMPs by the alveolar macrophages induces the production of the inflammatory cytokines: IFN-gamma, IL-12, IL-1β and macrophage inflammatory protein-1α [6, 16]. The main proinflammatory chemokines involved in the granuloma formation are TNF-alpha and IFN-gamma. The producers of the IFN-gamma are CD4+ lymphocytes (T helper 1), CD8+ lymphocytes (T suppressor) and natural killer cells. The role of IFN gamma consists in the activation of the macrophages and the production of the nitric oxide. The TNF-alpha is produced mainly by the CD4+ lymphocytes (T helper 1) and macrophages [23]. The TNF-alpha role in the granuloma formation consists in the activation of macrophages and chemokines production [15, 14]. The cytokine IL-10 is produced mainly by the B lymphocytes and AAMs [17]. The major role of IL-10 consists in the polarization of macrophages into the alternatively activated form [19]. The AAMs are induced by the cytokines IL-4 and IL-13, which are produced by the T helper 2 lymphocytes. The AAMs produce and secrete the IL-10, TGF-β and arginase [18]. The chemokine CXCL8 (IL-8) is a proinflammatory chemokine produced mainly by the macrophages and infected epithelial cells of the respiratory tract, which have the major role in the recruitment of the T lymphocytes (CD3+, CD4+ and CD8+ cells) and other immune cells into the infection site [18]. The ligation of the IL-8 to Mtb increases the ability of the neutrophils and macrophages to phagocyte and to kill bacilli [24].

Under the influence of the chemotactic agents, such as IL-1, IL-6, IL-8 and TNF-alpha secreted by the innate immune cells, such organs as the liver, especially the parenchimal hepatocytes, increases the production and secretion of the acute phase proteins (APPs) [25, 26, 27]. Biochemically, the APPs are polypeptides. Other systems involved in the production of the APPs are endothelial cells, connective tissue and epithelial cells. The most important APPs are C-reactive protein, haptoglobin and serum amyloid [25, 27]. However, several types of APPs were identified and classified into positive and negative. The positive APPs are considered a part of the innate immune system produced with the aim to destroy or inhibit microbes [28]. The concentration of the positive APPs (C-reactive protein, mannose-binding protein, complement factors, feritin, ceruloplasmine, serum amyloid A, haptoglobin and fibrinogen) increases when the infectious stimulus appears [25, 26, 27, 29]. Other APPs proteins give a negative feedback on the inflammatory response: alpha-2-macroglobulin, serpins, coagulation factors [28, 26]. The coagulation factors are parts of the innate immune system due to the increasing vascular permeability for phagocytes (neutrophil granulocytes and macrophages) and chemotactic agents [26]. The maximum concentration of APPs is revealed within 24 to 48 hours after the inflammatory injury. The decline of the AAPs level is identified due to feedback regulations that will limit the concentration in 4-7 days after the initial stimulus, if no further stimulus occurs. If the hepatic receptors are triggered continuously, the high level of the AAPs can become chronic. Chronic infection, which is revealed in the long lasting tuberculosis process increases the concentration of the AAPs, however the level is lower than in an acute infection perceived in nonspecific infections [25, 26, 27, 29]. Following the evaluation of the scientific review, we could not find data about the serum concentration of the cytokines and acute phase proteins in tuberculosis caused by Mtb with different types of resis-

tance. The aim of the study was to assess the differences in the serum level of the pro-inflammatory chemokines IL-8, cytokine TNF-alpha, anti-inflammatory cytokine IL-10 and acute phase proteins: c-reactive proteins, ceruloplasmine and fibronogen in patients with drug susceptible and drug-resistant tuberculosis.

Material and methods

It was realised a prospective research evaluating the biomarkers of the immune system in 87 cases, of which 24 were new cases with drug susceptible pulmonary tuberculosis included in the 1st study group and 27 were MDR-TB patients which were included in the 2nd study group. The groups were compared between them and were compared with a control group (CG) composed of 36 healthy persons assessed according to the clinical and biochemical criteria. The research reported ethics committee approval (nr. 14 of 21/11/2017) and patients' consent was obtained. Patients were diagnosed in the medical specialized institutions of Chisinau during the period 01.01.2016-31.08.2016. Including criteria in the study group were: age more than 18 years patients diagnosed with pulmonary tuberculosis, "new case" type, the diagnosis was confirmed through the conventional microbiological methods (microbiological examination and molecular genetic test of the sputum). The study investigation schedule included information about sex, age, radiological aspects, microbiological patient's status, results of the drug susceptibility test, treatment regimen and adverse drug reactions. The including criteria in the control group were: age more than 18 years, conventionally healthy persons according to the clinical examination, blood test (complete blood count) and biochemical tests (liver transaminases, bilirubin test, hepatitis virus serological tests, HIV serology). The immunological investigation of patients was performed during the intensive phase of the treatment. The 36 healthy persons from the control group were investigated in ambulatory conditions.

The BosterBio manufecturer was chosen for the acquisition of the ELISA Kits and antibodies. The kits were provided with 96 wells per kit. For the assessment of the serum level of the IL-8 was used the ELISA Kits and antibodies for human IL-8 PicoKine. The assay results ranged from 7,8 pg/ml till 500 pg/ml of serum or plasma. The serum level of the IL-10 was used in the ELISA Kit Human IL-10 PicoKine with the assay range between 3,4 pg/ml till 250 pg/ml. The level of TNF-alpha was assessed using the kit Human TNF-Alpha PicoKine with the assay range between 7,8 pg/ml till 500 pg/ml. Statistical analysis was carried out by the comparative assessment of the quantitative and qualitative peculiarities of the selected patients using the Microsoft Excel XP programme. Accumulated material was systematized in simple and complex groups. For the testing of significant differences between the studied indices of the compared samples it was performed the statistic non-parametric T test at the significant threshold $p<0,05$.

Results

While distributing patients, according to the biological characteristics was established a similar rate of men and women in all three groups, with the predomination of men in the same proportion, which was accepted as a condition that permitted the comparability of the results between the selected samples. The same proportion of young persons aged less than 44 years was established in all groups. All enumerated conditions permitted the comparability of the laboratory data (tab.1).

Table 1

Segregation of patients in sex and age groups

Biological segregation	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		N (%)	N (%)	N (%)
Sex stratification	Men	14 (58%)	18 (67%)	24 (67%)
	Women	10 (42%)	9 (33%)	12 (33%)
Stratification in age groups	18-44 years	18 (75%)	21 (77%)	29 (81%)
	≥45 years	6 (25%)	5 (23%)	7 (19%)

Detected by passive way, using standard tools (microbiological examination and chest X-ray) for the investigation of the symptomatic patients, were 15 (62%) cases from the 1st SG and 17 (63%) cases of the 2nd SG. The main proportion of both study groups was constituted from the patients with pulmonary infiltrative TB: 22 (91%) in the 1st SG and 24 (89%) cases in the 2nd SG. Radiological investigations identified the lung destruction in all selected TB patients. Microbiological status was positive in all patients and drug susceptibility testing permitted their distribution according to the obtained drug resistance results. Standard treatment for drug-sensitive TB was administrated in patients from the 1st SG and standard treatment for MDR-TB in patients from the 2nd SG. There were no major adverse drug reactions identified in the selected patients.

The assessed proinflammatory biomarkers constituted the serum concentration of the chemokine IL-8 and the cytokine TNF-alpha. The ELISA assay established that the concentration of IL-8 was up to 13 times more elevated in the groups diagnosed with tuberculosis. The concentration was higher even in the MDR-TB patients where the statistical threshold was not achieved. The concentrartion of the cytokine TNF-alpha was up to 4 times more elevated in the MDR-TB group and two times more elevated in the drug-sensitive TB group compared with the healthy individuals included in the control group and achieved a high statistical threshold in both study groups compared with the control group. Additionally, it was established a more elevated concentration in the MDR-TB group compared with the drug-sensitive TB group, achieving a high statistical threshold (tab. 2).

The assessed anti-inflammatory biomarker constituted the serum concentration of the cytokine IL-10. The concentration was statistically more elevated in both study groups of patients with tuberculosis compared with the control group (tab. 3).

Table 2

The serum level of the proinflammatory cytokines in the patients with drug-sensitive TB and MDR-TB

Cytokines	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		M±SD	M±SD	M±SD
IL-8	Assay range pg/ml serum	15.54±9.37 ○	16.05±7,68 ◊	1.68±1.16
	Compared to the control group	1339	1383	100
TNF-alpha	Assay range pg/ml serum	141.03±66.15 ●○	278.93±247.91 ◊	65.77±12.09
	Compared to the control group	216	427	100

Note: Values are mean ± SD. The percentage was assessed comparing the study groups with the reference value of the control group (100%). Comparison between study groups – ● p<0.001, comparison between the 1st SG and CG – ○ p<0.001, comparison between the 2nd SG and CG – ◊ <0.001.

Table 3

The serum level of the anti-inflammatory cytokine IL-10 in the patients with drug-sensitive TB and MDR-TB

Cytokines	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		M±SD	M±SD	M±SD
IL-10	Assay range pg/ml serum	0.08±0.04 □	0.08±0.02 ▪	0.06±0.011
	Compared to the control group	133	133	100

Note: Values are mean ± SD. The percentage was assessed comparing the study groups with the reference value of the control group (100%). Comparison between the 1st SG and CG – □ p<0.05, comparision between the 2nd SG and CG – ▪< 0.05.

The serum concentration of some acute phase proteins in tuberculosis patients established a statistically higher concentration of the ceruloplasmin in the drug-sensitive TB group and mildly elevated concentration in the MDR-TB group. It can be explained by a longer evolution of the MDR-TB which contributed to an intensive negative feedback, which diminished the hepatic synthesis of the ceruloplasmin. The serum level of fibrinogen was statistically more elevated in both groups with tuberculosis compared with the reference value of the control group, without difference among groups. The concentration of the C-reactive protein was detectable in a three times more elevated concentration than the normal conventional threshold (less than 6 mg/dL). The investigation was not performed in the control group, due to the conventional negative results in the healthy individuals (tab. 4).

Discussion

While distributing the patients, according to the sex and age, it was determined the predomination of men at economic, reproductive age (18-44 years) in both study groups, as well as in the control group, which was accepted as a condition for the comparability of the results. Detected, using standard microbiological examination and chest X-ray investigation, were two thirds of both study groups. Similar data were obtained in the national studies [30, 31]. The majority of both study groups was diagnosed with pulmonary infiltrative TB with lung destruction. Microbiological status was positive in all patients and drug susceptibility testing permitted their distribution according to the obtained drug resistance results. Standard treatment for the drug-sensitive TB was administrated in patients from the drug-sensitive TB group and standard treatment for MDR-TB in patients from the MDR-TB group. The regimens were used according to the WHO recommendations [4, 32].

Our immune biochemical research established a similar high level of the chemokine IL-8 in patients with drug-sensitive TB and MDR-TB. Other studies established high levels of IL-8 in tuberculosis and infections with mycobacteria other than tuberculosis [21]. The research of Ameixa C. proved the down-regulation of IL-8 secretion from Mtb infected monocytes by IL-10 [33]. Our clinical study estab-

Table 4

The serum level of the acute phase proteins ceruloplasmine and fibrinogen in the patients with drug-sensitive TB and MDR-TB

Cytokines	Parameters	1st SG (N=24)	2nd SG(N=27)	CG (N=36)
		M±SD	M±SD	M±SD
Ceruloplasmine	Assay range mg/ml serum	911.31±210.71 □	852.11±256.1	724.3±27.8
	Compared to the control group	125	117	100
Fibrinogen	Assay range ng/ml serum	4.00±1.11 ●	4.12±0.87 ●	2.24±0.48
	Compared to the control group	178	183	100
C-reactive protein mg/dL		21.87±19.57	18.57±16.99	NA

Note: Values are mean ± SD. The percentage was assessed comparing the study groups with the reference value of the control group (100%). Comparison between study groups and the control group – □ p<0.001, comparison between the 1st SG and CG – ● p<0.05. NA – non available.

lished thirteen times higher concentration of the IL-8 compared with IL-10, without differences between the study groups and it was not proved the regulation of Il-8 on the IL-10 concentration.

The concentration of the cytokine TNF-alpha was 4 times higher in the MDR-TB group and two times higher in the drug-sensitive TB group compared with the healthy group. Elevated concentration of the cytokine TNF-alpha was identified in multiple studies, which proved the boosted capacity of the macrophages to phagocytose and kill Mtb, when level of TNF-alpha is increased [15, 14]. However, similar researches identifying the differences between the concentration of the TNF-alpha in the drug-sensitive TB and MDR-TB were not found. The concentration of IL-10 was two times higher in both groups of patients at an analogical threshold. However, the concentration of IL-10 was not elevated at a similar height as IL-8 and TNF-alpha. Some studies established that an increased IL-10 level contributes to the survival of the Mtb in the infected host [33, 15, 17, 34].

The serum concentration of the positive acute phase proteins in selected patients established a statistically higher concentration of the ceruloplasmine in the drug-sensitive TB and a non-statistically higher concentration in the MDR-TB group. While identifying high concetration of active phase reactants, fibrinogen and ceruloplasmine Cernat R.I. established a straight correlation between those two active phase reactants. His research established high concentration of the Cu, Fe and Zn and the Cu-binding protein – ceruloplasmin before the initiation of the treatment [35]. However, similar studies to show the differences among drug susceptible and MDR-TB patients were not performed. Because an important protein is involved in the protection against oxidative stress, ceruloplasmin could be appreciated more as antioxidant than as proinflammatory biomarker [36, 37, 29]. Fibrinogen was found in an increased concentration in both groups and was established as a biomarker of tuberculous process by multiple researchers [38]. C-reactive protein was elevated in both groups of patients without differences among them and was interconnected with the increased concentration of fibrinogen and ceruloplasmin [27].

Conclusions

1. The proinflammatory chemokine IL-8 assessed in the serum through the ELISA assay established that the concentration of the IL-8 was 13 times higher in patients with both types of tuberculosis, without differences among drug-sensitive TB or MDR-TB.

2. The proinflammatory cytokine TNF-alpha was two times higher in the drug-sensitive TB and four times higher in the MDR-TB, being obtained at a high statistical threshold between groups. It can be connected with the extensiveness of the destructive process.

3. The anti-inflammatory cytokine IL-10 was two times higher in both groups of patients compared with the healthy individuals without any differences among groups.

4. The serum concentration of acute phase proteins: fibrinogen and C-reactive protein was revealed in a statistically higher concentration in both groups of patients, compared with the healthy individuals, without any differences among groups.

5. Ceruloplasmine serum level was higher in the drug-sensitive TB and non-statistically higher concentration in the MDR-TB group.

6. The proinflammatory cascade is more activated, than the anti-inflammatory response, without differences between drug susceptible and MDR-TB. CXCL8 (IL-8) and TNF-alpha can be assessed as proinflammatory biomarkers of tuberculosis, with no regard to the drug susceptibility. The serum levels of the anti-inflammatory cytokine IL-10 and acute phase proteins are mildly increased, without differences between the drug-sensitive and MDR-TB.

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Declaration of conflict of interests
Nothing to declare