The spontaneous and stimulated nitroblue tetrazolium (NBT) tests in mononuclear cells of patients with tuberculosis

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Abstract

Purpose: The aim of this study was to evaluate the ability of the NBT reduction by non- and BCG-stimulated monocytes isolated from peripheral blood persons with pulmonary tuberculosis (TB) before treatment, after two-month antituberculosis therapy and in an inactive stage of this disease.

Material and methods: The spontaneous and induced NBT tests were done in 24 healthy individuals and 59 patients with pulmonary tuberculosis: 33 before antituberculosis treatment and 26 with inactive stage of TB. Mononuclear cells were isolated from peripheral blood by the Bøyum method and indentified by histochemical assay. The abilities of non- and BCG-stimulated monocytes of NBT reduction were estimated by the method according to Park with Szczylik modification.

Results: In an active state of TB and after 2 months treatment, the non- and BCG-stimulated monocytes capacity to reduce NBT was found to be significantly increased in comparison to controls. The NBT test parameters in the absence of cell stimulation and after administration of BCG were comparable in active TB and after two-month treatment. In an inactive TB, the ability of NBT reduction by non- and BCG-stimulated monocytes was comparable to the controls. The stimulation of mononuclear cells accompanied by the significantly higher capacity of monocytes to reduce NBT in controls and in TB patients with post-tuberculous changes in the lungs.

Conclusions: These results of the spontaneous and induced NBT tests adequately reflect the status of the host's specific reactivity during tuberculosis and can be simple, cheep and useful for a monitoring antituberculosis treatment.

Key words: NBT test, monocytes, tuberculosis.

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Introduction

Tuberculosis (TB) is still an important world health problem, and it is estimated that about one third of the earth's population has been infected with Mycobacterium tuberculosis [1,2]. Each year, there are about 2 million deaths due to TB [1].

It is still not clear why only approximately 1 in 10 of those infected will progress to active disease during their lifetime, however, only a minority of them possess a risk factor, such as advanced age, alcohol abuse, immunosuppressive therapy, diabetes or an acquired immuno-deficiency syndrom (AIDS) [3].

Tuberculosis is a disease known to be associated with various immunological alterations, including some changes in the cell- and antibody- mediated immunity [4]. The monocytes/macrophages are the key cells determining the host's response to Mycobacterium tuberculosis. The monocytes/macrophages phagocytize acid-bacilli. The effectiveness of this process depends on age, sex, genetic factors and the Fc and C receptors expression of monocytes [5-13].

The phagocytosis causes an increase in metabolic activity of cells, which express itself in an intensified consumption of oxygen, and activation of pentose cycle [8,14,15]. The results of the metabolic transformation is a partial reduction of oxygen and a release of a number of highly active chemicals, which can destroy bacteria [4,8,10,16-20].

An indirect marker of the consequences of the metabolic activity is the reduction of nitroblue tetrazolium (NBT) by monocytes [14,21,22]. The NBT test study in tuberculosis has

been rarely reported and the results were inconsistent [16--19,23,24]. To the best of our knowledge, this is the first such study on the monocyte capacity of tuberculosis patients of NBT reduction in an inactive tuberculosis.

Therefore, the aim of this study was to evaluate the ability of the NBT reduction by non- and BCG-stimulated monocytes isolated from peripheral blood of individuals with pulmonary tuberculosis before treatment, after two-month antituberculosis therapy and in an inactive stage of this disease.

Material and methods

Patients

With the approval of the Local Ethics Committee, fifty-nine unrelated patients with pulmonary tuberculosis: 33 patients before antituberculosis treatment and 26 with inactive stage of disease, were studied. In the group were 25 women and 34 men on the range age 20-73 years (mean: 41.1 ± 16.4). Patients with newly detected active TB were admitted at the Pulmonology Hospital in Sopot, Poland. A diagnosis of TB was established using standard clinical, radiographic, and bacteriological criteria [25]. The studied patients were at similar clinical stage and with similar localized disease on the initial chest radiographs (CXRs) (the infiltrates with cavitation in one or two lung zones). The diagnosis of TB was confirmed in all patients by demonstration of acid-fast bacilli (AFB) in sputum smears and by positive sputum culture of the M. tuberculosis strains. The positive PPD skin – test was the additional criterion including to the study group. After a two month-therapy (rifampin, isoniazid, ethambutol, pyrazinamide), 12 patients were examined again and none of them showed the clinical evidence of active pulmonary tuberculosis. Response to therapy was defined as clinical and radiologic improvement with disappearence of AFB in the sputum on smear examination within 2 months from the start of therapy. The remaining patients were lost to follow-up. The other group included 26 patients who had documented completion of antituberculosis treatment and negative sputum smears for AFB and culture results, follow-up ranged from 1 to 17 years (mean, 14 years) in the Outpatient Tuberculosis Departments in Gdańsk. The current clinical examination and the CXRs (the tuberculous fibrotic scarring with caverns in the affected lung zones) excluded an active stage of the disease. None of these individuals relapsed during the period of follow-up. None of the patients had a family history of TB or other related diseases.

Controls

A total of 24 age- and sex-matched, unrelated healthy volunteers were included in the study as the controls. The individuals were screened and recruited from our University staff. Based on detailed clinical history, controls did not have a history of TB. None of them exhibited attributes of active TB or other related diseases based on clinical examination.

Tuberculin skin test

All tuberculin tests were done by the Mantoux method using 2 TU of human PPD Rt_{23} with Tween® 80 (Statens Sevminstitut, Copenhagen). The tests were read at 48 to 72 hours after administration. Two transverse diameters of induration being measured to the nearest millimeter. Any reaction ≥10 mm was considered a positive test [2].

Mycobacteriology

All tests were performed using standard methods that did not change during the study period. Direct sputum smears were examined by light microscopy and by cultures on 7H11, Löwenstein-Jensen medium. All cultures were identified as catalase/peroxidase – INH-sensitive M. tuberculosis strains by standard test [25]. The niacin production test for M. tuberculosis was used. The first culture with positive result was routinely tested for drug susceptibility to first-line drugs administered to all patients. No other biologic differences were observed in the M. tuberculosis strains.

CXR

The initial and follow-up standard posteroanterior chest radiographs of the patients and controls were reviewed. The CXRs were evaluated by two physicians who were blinded to the clinical data of the study population. The disease extent on the initial CXR was based on the number of lung zones involved; each lung was considered to have three zones (upper, middle, and lower). Involvement of one or two zones was considered to be localized disease. Follow-up CXRs were classified as showing improvement, progression, or no change. The presence or absence of infiltrate and/or cavitation was noted.

Assays performed

Isolation of mononuclear cells from peripheral blood by Bøyum method [26]: 10 ml of peripheral blood was put into test-tubes containing 14 mg of disodium-EDTA and 1 ml of 6% Dextran 500 (Pharmacia). After mixing the tubes were put into thermotast (37°C) for 40 minutes. Next the mixture was centrifuged 700 rpm for 10 minutes. The sediment was left aside and the received supernant was centrifuged 4 400 rpm for 30 min to obtain low platelet plasma, and then it was added to the previously remained sediment. This mixture was layered on 3 ml Nycodenz (Nycomed Pharma, Batch 710294), placed in a silicon test-tube and centrifuged at 600 xg for 15 min. The forming interface and the 2 mm region with monocytes lying below it were gathered with pipette and afterwards were washed with PBS. Then they were led to the volume of 1 mm and received suspension of cells with 95% of monocytes. The viability of the isolated monocytes was tested by 1% of trypan blue. Monocytes were indetified by histochemical methods [27]. The presence of brown granules in cytoplasma was identified as possitive result of this reaction. The results were performed after classification of each of 100 accepted cells recorded to a degree of intensification of the reaction. The results were given as a sum of products of all numbers with positive reaction multiplied by the degree of the reaction.

Evaluation of the reduction abilities of NBT by non-stimulated monocytes [22]: 100 μl of suspension of monocytes ($1x10^6$/ml), 100 μl of human serum AB Rh^+, 100 μl of PBS and 50 μl of NBT (Fluka AG, Buch SG: 6 mg/ml) were added consequently to a plastic test-tube. The mixture was incubated (37°C) for 15 minutes. Then it was cooled (4°C) for 3 minutes and

Table 1. **The ability of the NBT reduction test by non-stimulated monocytes in the tested groups**

Tested groups	No.	Mean in % ± SD
Controls	24	26.1 ± 6
TB patients before therapy	33	39.3 ± 14*
TB patients after 2-months therapy	12	38.6 ± 9#
Individuals with inactive TB	26	29.4 ± 7**†

TB – pulmonary tuberculosis
* – p<0.001 for the controls vs TB before therapy
# – p<0.001 for the controls vs TB after 2-months therapy
** – p<0.01 for inactive TB vs TB before therapy
† – p<0.01 for inactive TB vs TB after 2-months therapy

Table 2. **The ability of the NBT reduction test by BCG-stimulated monocytes in the tested groups**

Tested groups	No.	Mean in %± SD
Controls	24	33.8±9
TB patients before therapy	33	44.5±12*
TB patients after 2-months therapy	12	44.3±7#
Individuals with inactive TB	26	34.8±4**†

TB – pulmonary tuberculosis
BCG – bacillus Calmette-Guerin
* – p<0.01 for the controls vs TB before therapy
# – p<0.01 for the controls vs TB after 2-months therapy
** – p<0.01 for inactive TB vs TB before therapy
† – p<0.001 for inactive vs TB after 2-months therapy

centrifuged at 700 rpm/5 minutes. A cell smear was prepared and stained according to Mäy-Grünwald-Giemsa staining were done. Counting of monocytes of at least containing precipitated formazane were performed under immersion.

Evaluation of the reduction abilities of NBT by BCG-stimulated monocytes [28]: 100 µl of suspension of monocytes ($1x10^6$/ml), 100 µl of human serum AB Rh+, 100 µl of PBS, 10 µl (0.05 mg) of BCG and 50 µl of NBT (6 mg/ml) were added consequently to a plastic test-tube. The rest of the procedure as above.

Statistical analysis

Statistical analysis was performed by means of STATISTICA for Windows v. 5.0 (StatSoft) software using the Student-t test for groups comparison. Statistical significance was defined as $p \leq 0.05$. Data are presented as mean ($\bar{x}$) ± SD for each individual tested parameter.

Results

The ability of NBT reduction by non-stimulated monocytes in the tested groups is presented in *Tab. 1*.

The average percentage of test positivity by monocytes was significantly higher in an active TB as compared with the controls ($p<0.001$). A two-month treatment no change of NBT reduction by monocytes in comparison to the values in TB patients before therapy, but the values were higher than in the controls ($p<0.05$). In an inactive TB, the ability of NBT reduction was comparable to healthy people.

The ability of NBT reduction by BCG-stimulated monocytes in tested groups is presented in *Tab. 2*.

The average percentage of positivity of stimulated NBT reduction test by monocytes was significantly higher in an active TB vs the controls ($p<0.01$). After two-month treatment the values were not changed. In an inactive TB, the ability of NBT reduction by BCG stimulated monocytes was similar like in the group of healthy people.

The comparison of the ability of NBT reduction by non-stimulated and BCG-stimulated monocytes in tested groups is presented in *Fig. 1*.

In the group of patients with active TB, the values of the NBT reduction tests by non-stimulated and BCG-stimulated monocytes remain at the same level. A two-month treatment did not change the ability of spontaneous and stimulated NBT reduction in comparison to the values in TB patients before therapy. However, in the controls ($p<0.01$) and in inactive TB ($p<0.05$) the values of the NBT test by BCG-stimulated monocytes significantly increase as compared with the ability of reduction by non-stimulated monocytes ($p<0.01$; $p<0.05$, respectively).

Discussion

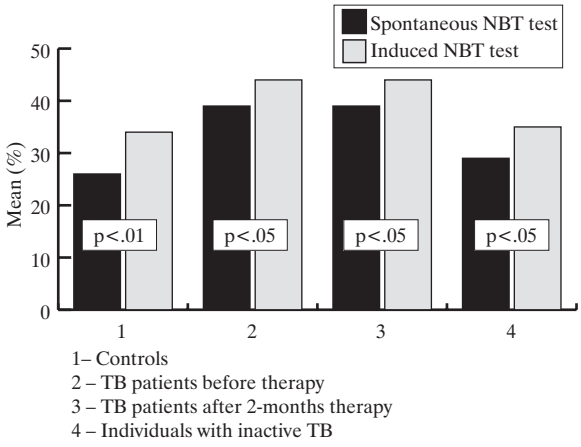
The nitroblue tetrazolium (NBT) test is an indirect marker of the oxygen-depend bactericidal activity of the phagocytes [4,8,10,14,20].

NBT is a dye with low reduction potential and performs intensively stained products–formazanes. NBT is easily phagotized by cells and is reduced to formazane inside mitochondrium.

The spontaneous NBT test use for screening of metabolic activity of granulocytes and/or monocytes. The induced NBT test assess the functional abilities of phagocytizing cells. The positive results of NBT reduction test show the percentage of stimulated cells by bacterial products phagocytes and occurrence of infection. The false positive results of NBT test exist during bacterial infection with presence of large necrosis or in limphoblastic leukaemia. False negative are present in myeloma [22].

The NBT reduction test and its modifications are useful for detection and differention of bacterial diseases. The NBT test on the granulocytes and monocytes can be used for the indirect evaluation of their bactericidal potency in non-specific infections but there are few inconsistent observations in tuberculosis [16-19,23,24,29]. Gracheva et al. [19] and Kaminskaja et al. [16,18,19,29] demonstrated the increasing insensitivity of the NBT reduction, whereas Garbiński et al. [23] and Shatrov et al. [24] revealed decreased abilities of nitroblue tetrazolium reduction by granulocytes, monocytes in an active tuberculosis. These cells, though capable of bactericidal action, do not react to stimulation Mycobacterium tuberculosis after 4 weeks of beginning of TB therapy [19,29]. To the best of our knowledge,

Figure 1. **The comparsion of the abilities of NBT reduction by non-stimulated nad BCG-stimulated monocytes in tested groups**



no report about the evaluation of the abilities of the NBT reduction by monocytes in an inactive tuberculosis.

According to Gracheva et al. [19] and Kaminskaja et al. [29], our results show the increased NBT reduction properties of monocytes both in an active tuberculosis and after two-month treatment. This increase of the ability of reduced of nitroblue tetrazolium can be a result of involvement of the oxygen-dependent mechanism of bactericidal activity of monocytes. The positive results of NBT tests after two-month treatment may suggest the presence of mycobacterial stimulation of monocytes or/and occurrence of necrosis in the affected tissue. The analysis of the spontaneous and induced NBT suggests a decreased of monocytes energetic reserve during additional stimulation of Mycobacterium tuberculosis in an active TB. However, monocytes isolated from healthy people and individuals with an inactive TB had greater abilities of bactericidal reduction after BCG stimulation in vitro.

Conclusions

These results of the spontaneous and induced NBT tests adequately reflect the status of the host's specific reactivity during tuberculosis and can be simple, cheep and useful for a monitoring of antituberculosis treatment.

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