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Quantitative assessment of tumor necrosis factor induction by the whole blood cells ex vivo, in the presence of Mycobacterium tuberculosis antigens

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We studied an efficacy of whole-blood method of tumor necrosis factor-alpha (TNF-alpha) induction in the presence of Mycobacterium tuberculosis antigens (tuberculin PPD and a mixture of specific recombinant ESAT-6 and CFP-10 antigens), for differential diagnosis of active tuberculous infection. A customized test system with immunoenzyme assay was developed, to quantify TNF-alpha in plasma samples. Based on findings of blood sample investigations in 46 adolescents with tuberculous infection (latent infection) and active pulmonary tuberculosis, the study demonstrated the possibility of differentiation between active and latent tuberculous infection, using ESAT-6 and CFP-10 antigens as TNF-alpha inducers.

Keywords: children, adolescents, tuberculosis, tumor necrosis factor, antigens

INTRODUCTION

The purposes of the use of immunodiagnostic methods in the field of tuberculosis (phthisiatry) are: diagnosis and differential diagnosis of tuberculosis, precise determination of the extent of tuberculosis activity, differentiation between postvaccinal and infectious allergies, and monitoring of the treatment efficacy and prediction of the disease course and outcome.

Reactivation of tuberculous infection seen in patients with rheumatoid arthritis during the treatment with Infliximab (trade name Remicade) humanized monoclonal antibodies is a vivid demonstration of the importance of TNF-alpha in the execution of antituberculosis immunity [3]. TNF is responsible for the formation and maintenance of granuloma that restricts the spread of tuberculous infection [4]. But, apparently, TNF-alpha is involved into the antimycobacterial resistance through a far more complex mechanism. $CD8^+CD45RA^+$ effector T-cells that ensure granulysin-mediated lysis of mycobacteria-infected macrophages have been shown to carry TNF-alpha molecules on their cell membranes. And this implies the lysis of these cells and blockade of the antimycobacterial effect by anti-TNF-alpha antibodies via the complement system [1]. Levels of antigen-stimulated TNF-alpha, unlike the levels of IFN-gamma, are not depressed in pulmonary tuberculosis with an extensive disease process [2], with the higher concentrations during active tuberculous infection, then during latent infection [8].

So the aim of the present work was to analyze the antigen-stimulated production of TNF-alpha in whole blood during different forms or phases of tuberculous infection, and during pulmonary diseases of a non-specific nature as well.

Materials and methods. The following groups of adolescent patients aged 14 to 17 have been studied (n=46): 8 patients with primary MTB infection (tuberculin test conversion); 10 patients with less then 2 years back history of recent MTB infection, adolescents with positive

Mantoux test; 20 patients with active pulmonary tuberculosis; 8 patients with localized forms of tuberculosis in the solidification and calcification phase.

Determination of antigen-stimulated TNF-alpha production. The whole blood for analysis was obtained in specimens of 3.5 mL each, with 20 U/mL of heparin, which were distributed to 3 tubes (5-mL sterile screw-top cryotubes), 1 mL per tube. One of the tubes was used as control, each of the 2nd and the 3rd tubes contained 10 mcL of PPD antigen or a mixture of ESAT-6 and CFP-10 recombinant antigens, respectfully. The antigens were added in doses of 10 μg protein. The percentage of specific recombinant peptides binding to cellulose-binding domain in the total pool of antigens added was nearly 20%.

An immunoenzymometric test system was developed to quantify TNF-alpha in supernatant plasma of the samples after whole blood incubation. Polyclonal rabbit antibodies produced by five-fold (at an interval of 2 weeks) immunization of rabbits with recombinant human TNF-alpha were added as a sorbent to the wells of polystyrene plates. Rabbit IgG were prepared by means of affine chromatography, using 'Protein A Sepharose FF' column. Direct ELISA titers of anti-TNF-alpha antibodies were shown to be at least 1:200 000. Anti-TNF-alpha F10 monoclonal antibodies [6], labeled with biotin and purified by 'Protein A Sepharose FF' column, were used as the detecting antibodies. Detection was made using streptavidin-peroxidase (Sigma), with orthophenylenediamine (OPDA) as a substrate for peroxidase.

Calibration curve was built based on consecutive double dilutions with standard recombinant TNF-alpha at concentrations ranging from 23.3 ng/mL to 0.05 ng/mL. 50 μL of diluting liquid and 50 μL of plasma samples were added to the wells on repeats.

Quantitative measurement of the responses was done using a 'Pikon' ELISA analyzer (Russia).

Assay sensitivity was 0.09 ng/mL. TNF-alpha levels in all antigen-stimulated samples were measured to be significantly higher than the test system sensitivity level.

Statistical assessment of inter-group differences was made by means of SPSS software with the use of a parametric Student's t-test and a nonparametric Wilcoxon test.

Results and discussion. Study results on antigen-stimulated TNF-alpha production in 46 adolescent patients are presented in Table 1.

According to the findings (Table 1), adolescents with suspected primary MTB infection had significantly higher levels of PPD-stimulated TNF-alpha concentration, then patients with recent infection or effectively treated patients with relatively minor forms of tuberculosis (primary tuberculous complex; infiltrating pulmonary tuberculosis, not extending beyond a single segment, in solidification phase). Similar findings between these forms of tuberculosis infection

were seen when TNF-alpha production was stimulated by a mixture of ESAT-6 and CFP-10 antigens, but the findings showed also that the levels of induced TNF-alpha were significantly higher in patients with active pulmonary tuberculosis (6.2±0.6 ng/mL) than in patients with primary infection (3.0±0.3 ng/mL).

Table 1.

TNF-alpha induction in whole blood samples incubated with MTB antigens

Patient groups	TNF-alpha concentration, ng/mL			
	Tuberculin PPD	p	Mixture of ESAT-6 and CFP-10 antigens	p
1. Newly identified MTB infection, n=8	3.8 ± 0.6	$p_{1-2}<0.01$	3.0 ± 0.3	$p_{1-4}<0.01$
2. Recent MTB infection, n=10	0.8 ± 0.2		1.4 ± 0.2	$p_{1-2}<0.01$
3. Active pulmonary tuberculosis, n=20	3.85 ± 0.6	$p_{3-4}<0.05$	6.2 ± 0.6	$p_{1-3}<0.01$ $p_{3-4}<0.001$
4. Minor forms of tuberculosis in solidification and calcification phase, n=8	1.45 ± 0.28	$p_{2-4}<0.05$	2.2± 0.3	$p_{2-4}=0.02$

The presented data indicate, that the level of TNF-alpha production in response to antigen stimulation reflects the activity of tuberculous infection. TNF-alpha was induced at very high levels in patients with fibrotic-cavitary pulmonary tuberculosis (14.1 ng/mL), and in patients with infiltrating tuberculosis concurrent with exudative pleuritis (9.13 ng/mL). Patient A, who had an infiltrating pulmonary tuberculosis concurrent with rheumatoid arthritis and was receiving Remicade (Infliximab) – a medication containing specific anti-TNF-alpha antibodies – although developed some observable TNF-alpha in response to PPD and a mixture of ESAT-6 with CFP-10, but at low concentration levels: 0.25 ng/mL and 0.6 ng/mL, respectfully.

The possibility of differentiation between latent and active infection based on TNF-alpha induction using PPD tuberculin as a stimulator, has been first demonstrated by Stern J.N. et al. [8].

Our findings, indicating the presence of significant differences between patients with active tuberculosis disease and non-active tuberculosis forms, correlate with the recently published reports by several authors [2, 5, 7-9] who found that differences exist between active and latent tuberculous infections, and that antigen-stimulated production of TNF-alpha decreases with effective treatment and mycobacterial expectoration cease. It is clear, that a very important set of data that will help to assess the clinical course and predict the outcome can be provided by immunological monitoring, such as the assessment of a specific immune response during disease and treatment, using a customized test system that would enable assessment of antigen-stimulated production for a number of cytokines.

We noticed, that levels of antigen-stimulated TNF-alpha remained high in patients who completed the intensive treatment phase, and a number of patients from non-active tuberculosis group who had profound residual effects after tuberculosis likewise had higher levels, compared to the rest of their group.

In fact, the development of a similar novel laboratory approach suitable for wide-scale clinical practice is required in surveillance for possible activation of tuberculous infection in HIV-infected patients with post-tuberculosis effects in the lungs. In this respect, a Luminex method could be a prospective automated technique for determination of cytokines, including TNF-alpha, in the antigen-stimulated blood samples, that could substantially improve the efficacy and specificity of this approach used for control of tuberculous infection.

Conclusions. The study results indicate that an ex vivo determination of antigen-stimulated tumor necrosis factor-alpha in whole blood samples can be used in diagnosing the tuberculous infection with the purposes of:

- differentiation between active or latent tuberculous infection;

- immunological assessment of the extent to which the disease is cured;
- differential diagnosis of tuberculosis and non-specific lung diseases.

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