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Immunological Analysis of Active Tuberculosis Infection in Children and Adolescents

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Abstract

The latent tuberculosis infection worldwide is determined by about 1.7 billion people. Identification of latent tuberculosis infection and prevention of the development of the disease is one of the highest priority tasks in the global radical reduction of the burden of tuberculosis. The research is devoted to the study of the effectiveness of cellular and humoral antigen-specific immunological methods for determining the initial signs of tuberculosis infection activity when examining children and adolescents with a latent form of this infection. Cellular immunological reactions in contrast to the determination of specific antibodies of blood serum using the recombinant specific protein ESAT-6-CFP-10, were not sufficiently effective in differentiating active and latent infections.

Determination of antibodies to the ESAT-6-CFP-10 protein with analysis of the specific activity criterion and determination of an increased concentration of neopterin up to 9 nM/L in blood plasma samples made it possible to reveal signs of tuberculosis infection activity. These results have been confirmed by the results of chest computed tomography of children and adolescents with the determination tuberculosis of intrathoracic lymph nodes or small intrapulmonary (2-5 mm) intrapulmonary foci, including those with signs of calcification.

Keywords: Latent TB Infection, Immunological Methods, Signs of Infection Activity, Children and Adolescents

Introduction

Implementation of a solution to the global problem of a radical decrease in the incidence of tuberculosis by 2035 requires rapid identification and treatment of active tuberculosis infection, as well as identification of latent tuberculosis infection and prevention of the development of the disease [2, 5, 7, 17].

Timely and accurate diagnosis of tuberculosis infection, as well as the determination of its activity are necessary conditions for reducing the incidence and prevalence of tuberculosis. The prevalence of latent tuberculosis infection in the world is 1.7 billion people [2, 5, 12, 15, 17]. Diagnosis of latent tuberculosis infection due to the absence of accurately identified clinical or radiological signs is provided by immunological tests in vitro or: intradermal tests, including the use of the Mantoux test. The use of recombinant proteins specific for M. tuberculosis as intradermal tests or using in vitro cellular reactions provides a significantly higher specificity of the results of diagnosing tuberculosis infection. The specific immunological tests in vitro, based on the assessment of antigen-induced production of interferon-gamma (IFN-γ) Quantiferon-TBGold In-tube, T-SPOT-TB (IGRA tests) [2, 7, 9, 25], Russian test the "Tubinferon" system [29, 30], as well as intradermal tests with recombinant tuberculosis antigen (ATR), commercial name -"Diaskintest" and its European analogue "C-tb" [1] are able to determine latent tuberculosis infection.

It has been hypothesized and discussed that high levels of specific immune response in interferon-gamma induction tests or in specific skin tests reflect active tuberculosis infection [10,18, 32]. According to new data from a meta-review of 34 publications [18], the risk of active tuberculosis infection increases only at very high concentrations of antigen-induced interferon-gamma, exceeding the level of a positive result by tens of times. Methods of cellular immunophenotyping are also discussed to differentiate active and latent TB infection [19, 22]. In case of positive results of these tests in children and adolescents in the absence of microbiological and radiological data in favor of active tuberculosis infection, it is recommended to prescribe chemo preventive treatment [5, 7, 14, 17, 25, 27, 34]. The most common chemoprophylaxis regimen is a 3-month course with isoniazid and rifampicin, and for patients with latent tuberculosis infection from family contact with a tuberculosis patient - a six-month course of chemoprophylaxis is prescribed, the drugs of which, however, have hepatotoxicity. Assessment of the activity of tuberculosis infection in children and adolescents presents particular difficulties due to the absence in most cases of isolation of Mycobacterium tuberculosis (MTB) in sputum samples and the absence of strictly defined X-ray criteria for determining active tuberculosis of the chest organs.

Differentiation of active and latent tuberculosis infection in children and adolescents is one of the fundamentally important tasks, according to a number of authoritative international publications [8, 20, 31].

In this regard, there is a great need to develop new biomarkers for determining cases of tuberculosis or predictive biomarkers for determining active tuberculosis infection in children and adolescents.

Despite the fact that it is cellular immunity that is able to control the multiplication of MTB, the attitude towards the role of humoral specific antibodies has changed in recent years. The inflammatory profile of specific antimycobacterial antibodies can be associated with various conditions of tuberculosis infection. [3, 21].

In recent years, molecular biological methods have been actively developing, primarily the search for promising genes, the expression of which would allow determining the activity of tuberculosis infection or assessing the prognosis of its activation. At the same time, some studies suggest transcriptome analysis of a fairly large number of genes [6, 13, 16, 23, 26]. Of great interest in prospective studies is aimed at determining the predictability of active tuberculosis infection among persons with home contact with tuberculosis patients.

As been shown before, on a relatively small number of samples in the study of the expression of the pdcd1 gene encoding the PD1 T-lymphocyte receptor, a high level of sensitivity for determining active tuberculosis infection and its differentiation from latent in-

fection in adolescent children was shown - 95.8% with a specificity of 94.4%; the area under the curve of statistical analysis according to the criteria of sensitivity - specificity - 0.96 [11].

Objective of the Study

Development of a complex of immunological methods for differentiating active and latent tuberculosis infection in children and adolescents and determining the signs of active tuberculosis infection when examining patients with a latent form of this infection.

To determine latent tuberculosis infection according to WHO recommendations in various countries, both a skin tuberculin test and the determination of specific induction of interferon-gamma in vitro were performed with used of the recombinant protein ESAT6-CFP-10 (Quanti Feron TB Gold. In Tube) [2, 7, 9, 10], or a skin test using this protein - "Diaskin-test" (in Russia) or C-tb [1] in European countries. At the same time, the use of the recombinant proteins demonstrates significantly more specific results for the determination of latent infection.

If the tests are positive, chemo preventive therapy is prescribed, mainly to persons from family contact with patients with tuberculosis.

In Russia, children and adolescents with a positive specific skin test "Diaskin-test" and in order to determine or exclude active tuberculosis infection undergo an X-ray examination - computed tomography of the chest organs and in the absence of obvious X-ray signs of pulmonary tuberculosis, are carried out within 2- 3 months preventive course of chemotherapy with two anti-tuberculosis drugs (isoniazid and rifampicin). At the same time, the drug sensitivity of the MTB that infected the child to these drugs is usually unknown, but the hepatotoxicity of these drugs is known. WHO also recommends for countries with a relatively high incidence of tuberculosis (less than 100 per 100 thousand population) to conduct at least 3-4-month chemo preventive courses of treatment of latent tuberculosis infection using rifampicin and isoniazid, or long-term, up to eight months courses isoniazid treatment, especially in tuberculosis contact groups and in patients with HIV infection [12, 17, 27].

In this regard, the development of immunological, as well as molecular biological methods for determining the signs of determining the reactivation of latent tuberculosis infection or the prognosis for the development of active infection, which would make it possible to identify a group of patients with a high risk of developing tuberculosis among children and adolescents with latent infection, is one of the main trends of modern scientific research [13, 16, 24, 33].

Materials and Methods

Patient Groups

A study of two main groups of patients was carried out: 1st group - 63 patients: children and adolescents of the inpatient department of tuberculosis of children and adolescents "NMITs FPI" of the Ministry of Health of the Russian Federation with signs of local pulmonary tuberculosis without bacterial excretion: mainly patients with tuberculosis of the intrathoracic lymph nodes; on two cases of focal and infiltrative pulmonary tuberculosis without destruction. 2nd group - 132 children and adolescents with signs of latent tuberculosis infection based on positive results of a specific skin test with recombinant tuberculosis antigen (ATR), commercial name "Diaskin-test", observed in the advisory department of the Moscow Regional TB Dispensary. At the same time, in the patients of this group, at the beginning of the studies, according to the results of clinical and X-ray examination, signs of active pulmonary tuberculosis or lymph nodes were not detected.

Criteria for infection with *Mycobacterium tuberculosis*: positive result of the specific skin test "Diaskin-test"; quantitative determination of antigen-specific induction of interferon-gamma (IFN-γ) using reagent kits: QuanttiFeron TB. Gold In Tube (Germany), as

well as the “Human interferon-gamma” kit (Invitrogen) in blood plasma samples in vitro after 24 hours incubation in the presence of a specific recombinant protein ESAT-6-CFP-10.

This protein was obtained in our laboratory together with the Russian research and production company Mon A LLC, which previously registered the “Tubinferon” reagent kit [30]. The criterion for a positive result was the determination of the concentration of interferon gamma at least 14 pg/ml.

Methods

1. Determination of the cellular immune response according to the criteria of antigen-specific induction of IP-10 protein and tumor necrosis factor TNF-alpha using the “Human IP-10 Instant ELISA” and “Human TNFalpha ELISA kit” reagent kits after incubation with highly purified ESAT-6 protein -CFP-10.
2. Quantitative determination of the neopterin molecule in blood plasma samples of the patients included in the study, not induced by antigens using the “Neopterin ELISA” reagent kit manufactured by “Invitrogen”.
3. Determination of antibodies to MTB specific antigenic complex ESAT-6-CFP-10 in serum samples of patients.

Determination of Serum Antibodies to Specific Protein ESAT-6-CFP-10

Preparing an Antibody Plate: Antigen (ESAT-6-CFP-10) at a concentration of 1 mg / ml was diluted 1: 200 in a carbonate-bicarbonate buffer solution pH 8.6 to obtain a final concentration of 5 μg / ml and applied to the wells of a flexible 96-well plate (Titertek) 100 μl per well. The antigen was incubated for 2 hours at 370 C; then the plate was washed four times, with an interval of 1 min., adding 200 μl of a "washing" solution to each well of the plate: phosphate-saline (0.02 M sodium phosphate in 0.85% NaCl) buffer solution pH 7.2 containing Tween 20 in dilution 1: 200). Then the wells of the plate were covered with 2% BSA protein (bovine serum albumin) and incubated for 1 hour at room temperature, after which they were washed once with distilled water, shaking out the remaining water.

Analysis and Accounting of Results: Serum samples were diluted 1:40 using phosphate buffered saline containing 2% bovine serum albumin (“dilution” liquid). Sera were added in 100 μL per well of the plate (duplicating). A pool of sera from healthy donors not infected according to the results of studies of antigen-specific induction of interferon-gamma was used as a negative control of the studied sera. Then the sera were incubated in the plate under the lid for 1 hour at 370 C, after which they were washed with the “washing” solution four times and added the conjugate: A protein-peroxidase diluted with the “diluting” liquid (1: 10000), 100 μL per well, which provides binding of peroxidase with specific antibodies immunoglobulin. Incubated for 30 minutes at 370 C and washed four times with "washing" solution. The results of the enzyme immunoassay were standardly developed using a substrate buffer solution containing hydrogen peroxide in a mixture with tetramethylbenzidine (TMB), with the development of staining, and using a stop reagent (sulfuric acid), stopping the development of the enzymatic reaction and taking into account the spectrophotometric absorption at 450 nm using the program computer "Zemfira", calculating the concentration of the investigated substance on the Bio-Rad Microplate Reader.

The determination of neopterin was carried out using the "Neopterin ELISA" "Gen Way" reagent kits based on the use of solid-phase competitive enzyme-linked immunosorbent assay using rabbit antiserum to neopterin, in which the results of the enzyme-linked immunosorbent reaction are taken into account in inverse proportion to the determined level of optical density measured on an enzyme immunoassay analyzer. For the studies, calibrators with certain concentrations of neopterin and a calibration curve with geometric proportions were used.

Results

The antigen-specific induction of interferon-gamma, as follows from the data presented in Table 1, was positive in cases of active tuberculosis infection in 95% of cases, while in latent infection, determined using the same specific skin test "Diaskin-test", it was in a slightly smaller percentage of cases. In the group of patients with latent infection, the percentage of positive results for determining a significant level of IFN-γ was slightly lower - 69.7%. This can be explained by the conduct of chemo preventive courses with the help of two anti-tuberculosis drugs: isoniazid and rifampicin.

The values of antigen-specific induction of interferon-gamma of these groups did not differ statistically. The levels of antigen-induced other important criteria of the immune response in tuberculosis infection: tumor necrosis factor (TNF-alpha) and IP-10 protein, which is presented in some works [20,27] as the most sensitive test for determining tuberculosis infection, were also not significantly different when comparing groups of patients with active and latent tuberculosis infection. It is possible that patients in the active group have already received specific therapy before admission to the hospital.

At the same time, we noted somewhat higher levels of antigen-induced TNF-alpha and IP-10 proteins in the group of patients with active tuberculosis infection.

The specific antibodies to the recombinant ESAT6-CFP-10 protein (table 2) in the group of patients with active tuberculosis infection were detected in 87.2% of cases. The index of antibody activity in the group with active infection was significantly higher than in patients with latent infection with an activity index significantly higher (7,7±0,96) than the level of antibodies in the group of patients with latent infection (4.4 ± 0.39) detected in 51% of cases.

However, the decrease in the antibody activity index noted after two months of specific treatment in patients with active tuberculosis infection was not significantly.

After examining the entire group of patients with latent tuberculosis infection using computed tomography of the chest organs, as well as a result of studies of the concentration of neopterin in whole blood plasma samples, a group of patients with a high risk of active tuberculosis infection was determined: 21 patients.

In this group, neopterin concentrations had higher neopterin levels (Table 3): from 9 nM / L or more. During clinical and radiological examination of these patients in the process of further observation, 5 patients were diagnosed with tuberculosis of the intrathoracic lymph nodes. In other patients, computed tomography of the chest organs revealed small intrapulmonary foci 2-5 mm in size.

Table 3 shows the results of studies of the concentration of neopterin in blood plasma samples in children and adolescents with pulmonary tuberculosis without bacterial excretion at the beginning of treatment and after two months of treatment. As shown in this table, neopterin concentrations in blood plasma samples were significantly reduced after two months of specific chemotherapy. In patients with latent TB infection, neopterin levels were significantly lower. These values were also significantly lower when compared with the group of patients with tuberculosis after 2 months of inpatient treatment.

However, as in the group of patients with small intrapulmonary foci found during examination of patients with suspected latent tuberculosis infection, as well as in 4 patients of the same group in whom tuberculosis of the intrathoracic lymph nodes was established during the examination, the concentrations of neopterin in plasma samples were comparable to the results of studies of neopterin in a group of patients with active tuberculosis infection.

In the same group, in the study of serum antibodies, their highest level was noted, determined by the criterion of the index of specific activity.

Groups	Number patients	IFN-γ, % positive results from 14 pg/ml		IFN-γ, (pg/ml)	TNF-α (пг/мл)	Student-test (t-test)	Protein IP-10 (pg/ml)	Student-test (t-test)
		Before treatment	After treatment					
1.Patients with pulmonary tuberculosis	63	95	66,6	116,4±24	223±28,6	P > 0,05	103,7±10	P >0,05
2. Latent TB infection	132	69,7		124,8 ±17	140 ± 38,2		74,6 ±2	

Table 1: Comparative analysis of antigen-induced cytokines groups

Groups	Number of patients	Antibodies to ESAT-6-CFP-10				Antibodies to ESAT-6-CFP-10/ activity index	
		% of positive results	Number of patients	Activity index	Student's test (t-test)	Active infection/ after treatment	Latent infection/ high risk group
1. Active infection/ pulmonary tuberculosis and TB of the intrathoracic lymph nodes	63	87,2	55	7,7±0,96	P < 0,034	6,96 ±0,54	10,2 ± 1,35; P< 0.01
2. latent infection	132	51	67	4,4±0,39			

Table 2: Specific antibodies to ESAT6-CFP-10

Groups		Number patients	neopterin concentration/ nM/l	Student's test (t-test)
Pulmonary tuberculosis and TB of the intrathoracic lymph nodes	At the beginning of treatment	43	16,5±1,17	
	After 2 months treatment	26	6,37±0,33	P<0,01
Latent of TB infection	With the presence of antibodies to ESAT-6-CFP-10	67	4,93±0,22	P<0,05
	Identified signs of TB activity	21	15,26±1,74	P<0,01

Table 3: Plasma neopterin concentration

The Discussion of the Results

The data obtained in the course of studies of patients with local tuberculosis infection and patients with latent infection (Table 1) on antigen-specific induction using a specific recombinant protein of mycobacterium tuberculosis - ESAT-6-CFP-10 - results of quantitative determination of cytokines: interferon-gamma (IFN -γ), tumor necrosis factor alpha (TNF-α) and IP-10 protein did not reveal significant differences between the two main groups. At the same time, somewhat higher numbers of antigen-induced TNF-α and IP-10 protein were noted in the group of patients with active infection compared with the group of patients with latent infection. However, these differences were not statistically significant.

Consistent with these results, the use of these criteria cannot be used to differentiate active and latent TB infection. It was also noted that the determination of a significant level of antigen-induced IFN-γ in the group of patients with active tuberculosis infection after two months of specific chemotherapy decreased from 95% to 66.6%. These results, in general, are consistent with the known studies on the decrease in the level of antigen-specific induction of IFN-γ after specific chemotherapy for tuberculosis infection [4]. In the group of patients with latent infection, the percentage of positive results for determining a significant level of IFN-γ was also slightly lower-69.7%, since patients with latent tuberculosis infection also previously received specific chemo preventive therapy.

As shown in Table 2, positive results for the determination of specific antibodies were determined in both compared groups: in 87.2% in the group with active infection and in a significantly smaller number of cases 51% in latent infection. The index of antibody activity in the group with active infection was significantly higher than in patients with latent infection.

As a result of the treatment carried out for two months in patients with active infection, the antibody activity index did not differ significantly from the initial values.

When analyzing the value of the activity criterion of specific antibodies, the most interesting phenomenon is the phenomenon of a high level of the index of specific activity of antibodies in a group of 21 patients with latent infection, in whom, in a detailed analysis of the results of computed tomography of the chest organs, signs of tuberculosis of the intrathoracic lymph nodes were found (4 patients), or small-point intrapulmonary cell clusters, in some of which there were signs of calcification. In this group, the index of specific activity of antibodies was the highest and most significant. In order to search for additional criteria of activity and discrimination of active and latent infection, the determination of the concentration of the neopterin molecule in blood plasma samples not induced by antigens was used.

Earlier in the work of Vasilyeva E.V. and co-workers [28] in studies of adult pulmonary tuberculosis patients with bacterial excretion and decay cavities, a very high level of neopterin concentration in blood plasma (more than 10 nM/l) was shown. A group of researchers [9] showed an increase in the level of neopterin in patients with rheumatoid arthritis receiving biological drugs that lower the immune defense factors in tuberculosis infection.

We also considered an increase in the level of neopterin in plasma not incubated with antigens as one criterion for assessing the activity of tuberculosis infection. For children and adolescents without signs of local forms of tuberculosis, the level of 9 nM / L was used as an activity criterion. Table 3 presents the results of studies of the concentration of neopterin at the beginning of treatment and after two months of treatment. The values of concentration of neopterin in blood plasma samples after the course of treatment were significantly ($P < 0.01$) and significantly lower. As noted above, patients with latent infection had the lowest levels of neopterin.

However, in 21 patients out of a total group of 132 patients with latent infection, who had chest CT scan revealed signs of tuberculosis of the intrathoracic lymph nodes (4 patients), or small intrapulmonary foci 2-5 mm. The average level of neopterin (15.26 ± 1.74 nM / L) in this group was comparable to the results obtained when examining patients in the active group of patients at the beginning of treatment.

Conclusion

1. The importance of determining the concentration of the neopterin molecule in non-antigen-induced blood plasma samples as one of the activity criteria - 9 nM / L was demonstrated, both for monitoring the effectiveness of treatment of children and adolescents with tuberculosis, and for determining the value of small-point intrapulmonary foci as signs of active tuberculosis. infections during examination of patients and differentiation of active and latent tuberculosis infection.

2. The research results demonstrate the importance of determining specific antibodies to the specific recombinant protein of mycobacterium tuberculosis ESAT-6-CFP-10, taking into account the analysis of their specific activity index to determine signs of activity when examining patients with latent tuberculosis infection.

3. The results obtained, in general, demonstrate the importance of immunological tests, in particular, the values of specific antibodies and their activity index, as well as the determination of the concentration of the neopterin molecule for differentiating active and latent tuberculosis infection, and suggest the prospect of a more accurate selection of a group of patients for longer courses of specific chemo preventive treatment.

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Conflict Interests

The authors declare no conflict of interest regarding the publication of this paper

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