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Comparison of Th1 and Th2 response in the blood of tuberculous patients and healthy contacts

Abstract

Introduction: Th1 response is known to play a dominant role in the resistance to tuberculosis. Nevertheless, IFN gamma levels are frequently increased in tuberculous patients, especially at the site of the disease. It is also possible that the shift toward Th2 response is responsible for the loss of resistance.

The aim of this study was to compare the Th1 function of peripheral blood cells and the levels of antimycobacterial antibodies in the serum of culture positive tuberculosis patients and healthy tuberculosis (Tb) contacts. The correlation between the levels of antimycobacterial antibodies and Th1 function of blood cells was also evaluated.

Material and methods: The material consisted of 51 tuberculous patients and 20 healthy persons, close contacts of tuberculosis patients. The ability of peripheral blood cells to secrete IFN gamma and IL-2 was estimated in whole blood cultures with PHA, PWM and tuberculin. The levels of IFN gamma and IL-2 in the supernatants of cultures was estimated via a commercial ELISA test. The levels of antimycobacterial antibodies was measured with commercial immunoenzymatic kits detecting IgG antibodies against 38 kDa+16 kDa and IgG, IgA and IgM antibodies to 38 kDa + lipoarabinomannan (LAM).

Results: No difference was found in the secretion of IFN gamma and IL-2 after stimulation with PHA and PWM between the patients and contacts. The secretion of IFN gamma after stimulation with tuberculin was even greater in tuberculous patients than in contacts. The levels of IgG and IgA (38 kDa+LAM) were higher in tuberculous patients than in contacts. There was a negative correlation between the level of IgG anti 38 kDa+LAM and the ability of peripheral blood cells to secrete IFN gamma after non-specific stimulation in patients with tuberculosis.

Conclusions: Our study confirms the hypothesis that it is not the diminished production of Th1 cytokines, but rather the parallel overproduction of Th2 cytokines, which are essential in the development of tuberculosis.

Key words: Th-1, Th2 response in tuberculosis, IFN gamma, IL-2, antimycobacterial antibodies

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Introduction

It is well known that only about 10% of people infected with *Mycobacterium tuberculosis* develop active disease. Many facts point to IFN gamma as the main cytokine responsible for this phenomenon, among others the great sensitivity to diseases caused by mycobacteria in patients with genetically-based disturbances of IFN gamma-IL-12 axis [1].

On the other hand, individuals with tuberculosis have a strong Th1 response, especially at the site of disease [2–4].

Thus, it is clear that IFN gamma is essential for protection. But it also seems that IFN gamma levels alone cannot explain the immunity/susceptibility dichotomy [5]. It is possible that Th1 response is only effective in the absence of another corrupting influence. This corrupting influence may be a form of Th2-like response, because IL-4

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down regulates inducible nitric oxide synthase [6]. However, the role of this cytokine may be controversial as we now know that it consists of two forms: IL-4 and its splice variant and antagonist IL-4 delta 2 [6].

Dheda et al. proved that mRNA levels of IL-4 and IL-4 delta 2 were elevated in unstimulated cells from blood and lung lavage of patients versus control subjects. The mRNA half life of IL-4, but not of IL-4 delta 2, was significantly prolonged in tuberculous patients [7]. Demissie et al. showed that healthy infected subjects exhibit a selective increase of message for IL-4 delta 2 [5]. Thus it seems that long-term control of *Mycobacterium tuberculosis* infection is associated not just with elevated Th1 response, but also with the inhibition of the Th2 response.

The Th2 response manifests itself, among other means, by elevated levels of antituberculous antibodies in the blood.

The aim of this study was to compare the Th1 function of peripheral blood cells and the level of antimycobacterial antibodies in the serum of sputum positive tuberculous patients and their contacts.

We were also looking for any correlation between Th1 response and production of antimycobacterial antibodies in tuberculous patients.

Material and methods

The study group comprised 51 patients with tuberculosis and 20 healthy workers at the Lung Diseases Hospital who had been in contact with culture-positive tuberculosis for a minimum of five years.

The group of tuberculosis patients consisted of 24 women and 27 men aged from 19 to 80 years (mean 45.2 ± 20.2). Contacts were 15 women and five men aged from 29 to 69 years (mean 45.8 ± 11.3).

The diagnosis of tuberculosis was proved by the presence of tubercle bacilli in sputum or in bronchial washings confirmed by culture and the presence of symptoms characteristic of the disease and typical radiological findings. Only patients without evident immunologic disturbance were recruited to the study group. Thus, all in whom tuberculosis was connected with diabetes mellitus, cancer, HIV or renal insufficiency, were excluded.

The cytokine production by peripheral blood mononuclear cells (PBMC) was estimated in whole blood cell cultures according to the method developed by Elsässer-Beile et al. [8] and applied also to tuberculosis patient's samples by Elliot et al. [9].

5 ml of heparinized blood was taken from healthy donors and from tuberculous patients before treatment. Cultures were performed over two hours after taking the blood in the standard medium with RPMI 1640 (GIBCO) supplemented with L-glutamine, streptomycin (50 μg/ml) and penicillin (50 μg/ml).

For stimulation, Phytolacca Americana (PWM--Sigma) in the concentration of 5 μg/ml, PHA (Murex) in the concentration of 10 μg/ml and tuberculin (RT Statens Serum Institute) in the concentrations of 12.5 μg/ml and 25 μg/ml were used.

In every test tube (6 ml Falcon 2058) 50 μl of blood, mitogen diluted in 50 μl of RPMI 1640 or 50 μl RPMI without mitogen and 400 μl RPMI were placed.

Every blood specimen was cultured in duplicates: samples with PWM,with PHA and without any stimulant. In addition, the blood from 25 patients with tuberculosis and 14 healthy contacts was cultured in duplicates with tuberculin in the concentration of 12.5 μg/ml and in the concentration of 25 μg/ml.

The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. After four days of incubation, supernatant was removed from each tube to be analyzed for cytokine levels. The levels of cytokine were assayed with a commercial ELISA test (R&D) with the range for IFN gamma 0––1000 pq/ml (detection limit 8 pq/ml) and for IL-2 range 0–2000 pq/ml (detection limit 7 pq/ml). After pilot experiments we have found that the optimal dilution of the supernatants for IFN gamma stimulation is 1:160, and for IL-2 1:10. The levels of cytokines were proportional to the optical density measured by ELISA with reader (ELX 800 Bio-Tek Instruments Inc.) at the wavelength 450 nm. The results were calculated using the standard curve. The final result was an arithmetical mean of the results from two replicates. In the same day 2 ml of blood was taken on EDTA for flow cytometry (BD Bioscenses FACScan) with programme SimulSET version 3.1 and for detection of antimycobacterial antibodies.

Blood samples for antimycobacterial antibodies were collected, centrifuged and serum was stored at –40°C until use. An array of commercial immunoenzymatic kits to detect IgG antibodies against 38 kDa plus 16 kDa (Pathozyme tb complex plus, Omega Diagnostics, Scotland) and IgG, IgA and IgM antibodies to 38 kDa plus lipoarabinomannan (LAM) (MycoG, MycoA, and MycoM, Omega Diagnostics, Scotland) was applied. 38 kDa and 16 kDa are recombinant mycobacterial antigens expressed in and purified from *E. coli*. LAM is native mycobacterial antigen. All tests are based on a solid double antibody sandwich ELISA.

Sera diluted 1:50 or 1:100 (according to manufacturer instruction) were added to microwells precoated with antigens. All samples were assayed in duplicates. In the positive cases, antigen-anti-

Table 1. White blood cell counts among patients with tuberculosis and healthy contacts

White blood cells population		Patients with tuberculosis	Healthy contacts	P value*
Sample		31	20	
Leukocytes	Median	8740	5900	0.0013
	Mean ± SD	8526.48 ± 2497.63	6344.5 ± 1991.40	
Monocytes	Median	543	418	0.0039
	Mean ± SD	575.1 ± 234.94	426.6 ± 146.24	
Neutrophiles	Median	5923	3236.5	0.0001
	Mean ± SD	5956.13 ± 2407.25	3656.7 ± 1517.36	
Lymphocytes	Median	1477	1979	0.0580
	Mean ± SD	1711.42 ± 711.18	1960.9 ± 555.22	
Lymphocytes B	Median	135.5	165	0.0973
	Mean ± SD	168.97 ± 141.58	182 ± 79.30	
Lymphocytes T	Median	1205	1468.5	0.0638
	Mean ± SD	1233.23 ± 559.73	1433.9 ± 419.88	
Lymphocytes T CD4	Median	746	813.5	0.0516
	Mean ± SD	704.32 ± 335.86	871.2 ± 338.24	
Lymphocytes T CD8	Median	463	521	0.1823
	Mean ± SD	496.77 ± 259.77	541.65 ± 201.56	
Lymphocytes T CD4/CD8	Median	1.48	1.45	0.3182
	Mean ± SD	1.63 ± 0.77	1.82 ± 0.94	
NK cells	Median	280	300.5	0.3244
	Mean ± SD	319.47 ± 188.43	338.9 ± 156.66	

*Differences between patients with tuberculosis and healthy contacts were analyzed using U Mann-Whitney test. Statistical significance was considered if the p value was less than 0.05

body complex reacted with peroxidas-labelled antihuman IgG (IgA or IgM) conjugate. Using H_2O_2/ /TMB as substrate, the enzymatic activity of peroxidase was measured at 450 nm with the use of automated reading system ELX 800 (Biotec).

All the results, except for IgM tests, were referred to the standard curve. The standards were provided for the generation of a semi-logarithmic reference curve. As the sera were diluted 1:50 or 1:100, the units extrapolated from the standard curve were multiplied by 50 (100) to obtain serounits for result interpretation. IgM test results were expressed as a ratio of optical density (OD) of the examined sample to the OD of the cut-off sample.

For statistical analysis, non-parametric test U Mann-Whitney was used. Additionally, the Spearman test was used to assess the correlation. The study was accepted by the Ethical Committee of the Institute of Tuberculosis. All subjects gave informed consent for participation.

Results

In patients with tuberculosis, significantly higher counts of leucocytes, monocytes and neutrophiles were found compared to healthy contacts. However, there were no differences in the number of lymphocytes, B, T and TCD4 (Table 1).

No difference was found between the two groups in the secretion of IL-2 and IFN gamma after stimulation with PHA and PWM (Tables 2, 3).

The secretion of IFN gamma after stimulation with tuberculin was significantly higher among patients with tuberculosis than in controls (Table 4).

The levels of IgG and IgA anti 38 kDa+LAM were higher in tuberculous patients than in contacts (Table 5).

In addition, a negative correlation between the level of IgG anti 38 kDa + LAM and the ability of peripheral blood cells to secrete IFN gamma after non-specific stimulation was found in tuberculous patients (Table 6).

Discussion

Our study estimated the cytokine secretion after stimulation with PHA, PWM and tuberculin in whole blood culture.

The use of whole blood culture was developed by Luquetti and modified by Elsässer-Beile [8]. The author compared whole blood culture with the culture of isolated mononuclear cells and found that there is a direct correlation

Table 2. IFN gamma production by whole blood cells from Tb patients and controls stimulated with PWM and PHA

IFNγ [pq/ml]		Patients with tuberculosis	Healthy contacts	P value*
Sample		51	20	
PWM	Median	33 985.9	43 027.6	0.1548
	Mean ± SD	77 354.8 ± 113 389.6	46 198.5 ± 19 992.2	
PHA	Median	41 010.0	36 571.0	0.1401
	Mean ± SD	86 831.7 ± 134 429.0	38 555.8 ± 23 681.5	

*Statistical analysis was performed with the use of Mann-Whitney U test. Statistical significance was accepted at the level of $p < 0.05$

Table 3. IL-2 production by whole blood cells from patients with tuberculosis and healthy controls stimulated with PWM and PHA

IL-2 [pq/ml]		Patients with tuberculosis	Healthy contacts	P value*
Sample		51	20	
PWM	Median	337.5	431.5	0.3250
	Mean ± SD	617.2 ± 659.7	524.9 ± 425.9	
PHA	Median	631.2	827.9	0.4669
	Mean ± SD	1676.7 ± 2221.9	1181.6 ± 1068.8	

*Statistical analysis was performed with the use of Mann-Whitney U test. Statistical significance was accepted at the level of $p < 0.05$

Table 4. Secretion of IFN gamma by whole blood cells from patients with tuberculosis and healthy contacts stimulated with PPD (RT25 μg/ml and 12.5 μg/ml)

IFNγ [pq/ml]		Patients with tuberculosis	Healthy contacts	P value*
Sample		25	14	
RT 25	Median	3670.81	1479.35	0.0141
	Mean ± SD	7289.54 ± 10 019.47	3154 ± 4794.88	
RT 12.5	Median	3141.98	774.94	0.0116
	Mean ± SD	7954.68 ± 15 856.46	5051.24 ± 11 846.58	

*Statistical analysis was performed with the use of Mann-Whitney U test. Statistical significance was accepted at the level of $p < 0.05$

Table 5. Antimycobacterial antibody levels (Pathozyme-Plus and Pathozyme-Myco G, -Myco A and Myco M) in sera from patients with tuberculosis and healthy contacts

Serological tests		Patients with tuberculosis	Healthy contacts	P value[a]
Sample		50	20	
IgG anti 38+16 kDa[b]	Median	171.6	147.2	0.2355
	Mean ± SD	349.0 ± 485.9	154.9 ± 80.4	
IgG anti 38 kDa+LAM[b]	Median	253.9	45.4	0.0155
	Mean ± SD	387.5 ± 500.7	82.5 ± 139.0	
IgA anti 38 kDa+LAM[b]	Median	365.6	152.7	< 0.0001
	Mean ± SD	936.6 ± 1792.9	187.8 ± 111.5	
IgM anti 38 kDa+LAM	Median	0.65	0.74	0.4650
	Mean ± SD	0.83 ± 0.52	0.77 ± 0.38	

[a]Statistical analysis was performed with the use of Mann-Whitney U test. Statistical significance was accepted at the level of $p < 0.05$
[b]IgG and IgA antibody level was expressed in U/ml. IgM antibody level was expressed as optical density ratio

between both methods in the assessment of cytokine secretion.

Our study found that IL-2 and IFN gamma secretion after stimulation with PHA and PWM was similar in blood from tuberculous patients and from healthy contacts. The comparable results were obtained after non-specific stimulation by Morosini et al. and De Castro-Cunha et al. by assessing the number of cells secreting IFN gamma [10, 11].

Table 6. Correlation between antimycobacterial antibody level and PWM or PHA-induced production of IL-2 and IFN gamma in blood from patients with tuberculosis

Patients with tuberculosis	PWM/IL-2	PHA/IL-2	PWM/IFNγ	PHA/IFNγ
Sample	50	50	50	50
IgG anti 38+16 kDa				
Spearman correlation	–0.1452	–0.0964	–0.0199	–0.0268
P value[a]	ns	ns	ns	ns
IgG anti 38 kDa+LAM				
Spearman correlation	–0.3358	–0.4462	–0.4553	–0.4707
P value[a]	*	***	***	***
IgA anti 38 kDa+LAM				
Spearman correlation	–0.0593	0.0022	–0.0195	–0.1114
P value[a]	ns	ns	ns	ns
IgM anti 38 kDa+LAM				
Spearman correlation	–0.0037	–0.1905	–0.1053	–0.1292
P value[a]	ns	ns	ns	ns

[a]The results are calculated with the use of Spearman correlation test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns — not significant

On the other hand, Garcia et al. [3] and Toossi et al. [12] have found diminished number of lymphocytes positive for Th1 cytokines after non-specific stimulation. The discrepancy of those results may be due to the selection of patients with different forms of tuberculosis. We found secretion of cytokines after non-specific stimulation was diminished in cases where the disease was far advanced [13].

In the present study, the production of IFN gamma after stimulation with tuberculin was significantly higher in tuberculous patients than in contacts. The results presented in the literature dealing with this problem are inconsistent. Many authors have found weaker production of Th1 cytokines after stimulation with specific antigens among tuberculous patients [3–5, 14–16]. Others have obtained different results. Długovitzky et al. [17] found that the production of IFN gamma, TNF alpha and TGF beta after stimulation with a sonical form of mycobacterium tuberculosis was increased in tuberculous patients in comparison to controls. Fortes et al. [18] found that the number of lymphocytes positive for IFN gamma after stimulation with ESAT-6 is higher in patients with non-resistant tuberculosis compared to healthy controls, and to patients with drug-resistant tuberculosis. The observation was noted by Ferrand et al. [19], by Ulrichs et al. [20] and by Morosini et al. [10] in cultures stimulated with tuberculin.

Many results point to the increased function of Th2 cytokines in tuberculosis. Many authors have found increased levels of IL-10 in the blood of tuberculous patients [2, 3, 5, 14, 15, 21]. Sanchez et al. [16] found elevated levels of IL-4 after stimulation with PPD together with high levels of antituberculous antibodies. Seah et al. [22], using reverse transcription polymerase chain reaction on freshly isolated peripheral blood mononuclear cells, found increased expression of IL-4 and IL-13.

We have found higher levels of antituberculous antibodies in the serum from tuberculous patients comparing to healthy contacts.

We have also found a negative correlation between the level of antimycobacterial antibodies in the blood and the secretion of IFN gamma and IL-2 in whole blood cultures after non-specific stimulation.

Conclusions

Our results are thus in concordance with the opinion of others: it is not the diminished production of Th1 cytokines, but rather the parallel overproduction of Th2 cytokines that is essential in the development of tuberculosis.

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