Increased Th1 and Th2 Type Cytokine Production in Patients with Active Tuberculosis

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Abstract

Background: The global spread of tuberculosis necessitates the development of an effective vaccine and new treatment modalities. That requires a better understanding of the differences in regulation of the immune responses to *Mycobacterium tuberculosis* between individuals who are susceptible or resistant to the infection. Previous immune studies in young Ethiopian immigrants to Israel did not demonstrate anergy to purified protein derivative or a Th2-like cytokine profile.

Objectives: To evaluate the profile of Th1 and Th2 cytokine production in immigrant TB patients, in comparison with asymptomatic control subjects.

Methods: The present study included (part 1): 39 patients with acute TB(group 1), 34 patients with chronic relapsing TB (group 2), 39 Mantoux-positive asymptomatic TB contacts (group 3), and 21 Mantoux-negative asymptomatic controls (group 4). Patients were mainly immigrants from Eastern Europe and Ethiopia. Levels of interferon gamma, interleukin 2 receptor, IL-6 and IL-10 were measured in serum and in non-stimulated and PPD-stimulated peripheral blood mononuclear cell culture supernatants, using commercial ELISA kits. In addition (part 2), levels of IFNγ and IL-12p40 were evaluated in 31 immigrant Ethiopian patients and 58 contact family members.

Results: Patients with acute disease tended to secrete more cytokines than contacts, and contacts more than chronic patients and controls, without a specific bias. None of the patients showed *in vitro* anergy. Discriminant probability analysis showed that from the total of 12 available parameters, a cluster of 6 (IFNγ-SER, IFNγ-PPD, IL-2R-SER, IL-10-SER, IL-10-NS and IL-6-PPD) predicted an 84% probability to become a TB contact upon exposure, 71% a chronic TB patient and 61% an acute TB patient. Family-specific patterns of IFNγ were demonstrated in the second part of the study.

Conclusions: Firstly, no deficiency in cytokine production was demonstrated in TB patients. Secondly, acute TB patients secreted more cytokines than contacts, and contacts more than unexposed controls. Thus, neither anergy nor a cytokine dysregulation explains susceptibility to acute TB disease in our cohort, although chronic TB patients produced less cytokines than did acute patients and less than asymptomatic contacts. Thirdly, a certain cytokine configuration may predict a trend of susceptibility to acquire, or not acquire, clinical TB. It is presently unclear whether this finding may explain the disease spread in large populations. Finally, the familial association of IFNγ secretion levels probably points towards a genetic regulation of the immune response to *Mycobacterium tuberculosis*.

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During the last decade tuberculosis has posed an increasing global threat [1] for which an effective vaccine is not as yet available, and mycobacterial drug resistance is spreading. A better understanding of the protective immune responses and the causes of susceptibility to the disease are essential for the development of efficacious vaccines [2] and a new generation of immune-targeted drugs.

Some countries suffer from a high incidence of TB, such as Ethiopia and Eastern Europe, from which growing numbers of immigrants have arrived in Israel during the last two decades [3]. Among these immigrants are tuberculous children, and several immune studies have been conducted in this group [4]. This presented the opportunity to expand the study of some immune parameters in patients from these populations in the acute and chronic stages of the disease, as compared to a sample from the indigenous control population that tested negative for skin-purified protein derivative. The cytokines chosen for evaluation were among those considered important in host responses to *Mycobacterium tuberculosis*, except for interleukin-2 receptor, the shedding of which is considered to be a non-specific marker of T cell activation. Furthermore, although we previously demonstrated that a cohort of young Ethiopian TB patients and contacts did not exhibit a cell-mediated specific immune defect, anergy to PPD or a Th2-type bias in the cytokine profile [4], we also report the results of an assessment of *in vitro* cytokine secretion in a group of Ethiopian immigrant TB patients and their non-affected close family contacts.

Patients and Methods

The population included in the first part of the study consisted of East European and Ethiopian immigrant TB patients

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TB = tuberculosis
IL = interleukin
PPD = purified protein derivative
IFNγ = interferon-gamma
SER = serum
IL-2R = interleukin 2 receptor
NS = non-stimulated

and contacts and Israeli resident controls, divided into four groups:

- Group 1: 39 acute TB patients presenting with cough and occasional fever, a positive chest X-ray, pulmonary infiltrates and/or cavities and/or mediastinal lymphadenopathy, a highly positive Mantoux test (> 15 mm), and positive sputum bacteriology in some cases.
- Group 2: 34 patients with chronic or relapsing pulmonary TB after treatment, some with lung cavitation and/or multiple drug-resistant bacterial strains.
- Group 3: 39 Mantoux-positive (> 10 mm skin reaction), asymptomatic known TB contacts.
- Group 4: 21 Israeli resident Mantoux-negative (< 5 mm) laboratory and nursing personnel, as controls.

For the first part of the study the patients were enrolled from a pulmonary TB inpatient unit (Shmuel Harofeh Hospital, Beer Yaacov). Participants in the family studies of the second part were referred for TB treatment from the Ambulatory TB Rehovot and Ashkelon Centers. These included 60 TB Ethiopian immigrant patients and 118 parents, siblings and/or offspring from 38 families, all of whom arrived in Israel within the year prior to inclusion in the study. All patients with active disease were in various stages of a three-to-four drug chemotherapy regimen (from 2 weeks to 6 months). Chronic/relapsing patients had already undergone more than one course of chemotherapy. Some contacts were receiving preventive isoniazid.

The Mantoux test

This text was performed by intradermal injection of 0.1 ml of 5 PPD tuberculin units and the largest diameter of the ensuing papule was measured after 48–72 hours.

Cytokine measurements

In the first part of the study cytokines were measured in serum and in supernatants of cultured PBMCs (2×10^6 cells/well) for 48 hours with or without 10 µg/ml PPD. Samples were stored at -70°C, then assayed for interferon-gamma, soluble interleukin 2 receptor, IL-10, IL-6 and the IL-12 heterodimer (IL-12p70). In the second part only IFNγ and IL-12 were evaluated. This time the level of IL-12p40 was measured due to the poor results obtained with the heterodimer.

Assays were performed using commercial kits (R & D Systems, Minneapolis, MN, USA) in a standard procedure, using a sandwich double-antibody,

PBMC = pripheral blood mononuclear cell
sIL-2R-NS = non-stimulated sIL-2R

enzyme-linked colorimetric technique, in which the optic density of the developed dye was compared to a standard optical density curve.

Statistical analysis

Statistical analysis was performed using the automated NCSS program. Significance of intergroup differences in the data was tested by the chi-square and unpaired t-tests. Discriminant analysis was done by the Mann-Whitney U-test.

Results

Values for cytokine levels for the first part of the study, in serum, unstimulated PBMC and PPD-stimulated PBMC are summarized in Table 1 and presented in detail in Figure 1 as mean ± standard deviation U/ml or pg/ml for each of the four groups, separately. P values for intergroup comparison were within 95% confidence limits. IL-12 was undetectable in most cases, probably due to the fact that the p70 heterodimer was measured, for which PPD may have been an insufficient stimulus. Under Table 1A the discriminant analysis of the differences between the groups is given [Table 1B]. sIL-2R-NS values in TB contacts and controls were below the detection level of 50 pg/ml, therefore intergroup analysis was not done.

The major findings were as follows: acute patients (group 1) had much more sIL-2R in their serum, as compared to chronic patients (group 2) ($P > 0.001$), as well as IL-10 in supernatants from unstimulated PBMC ($P > 0.05$) and in PPD-stimulated PBMC ($P > 0.01$). No significant difference in IFNγ and IL-6 levels was

Table 1. [A] Comparison of cytokine secretion between study groups (part 1). Group 1 patients (acute TB) had more sIL-2R in their serum than group 2 (chronic) and group 3 (contacts). Also, the cells of group 1 secreted more IFNγ than those of group 3, in response to PPD, as well as more IL-10 than in group 2. At the same time group 3 had more IFNγ, sIL-2R and IL-10 in the serum than group 2, while the cells of group 3 secreted more IFNγ and IL-10 than in group 4 (controls).

	IFNγ			sIL-2R			IL=10			IL-6		
	SER	NS	PPD	SER	NS	PPD	SER	NS	PPD	SER	NS	PPD
Active-Chronic Groups 1 and 2				***				*	**		*	
Active-Contacts Groups 1, 2 and 3		**		***			**					
Contacts-Chronic Groups 3 and 4	*			*			*	**	*		**	
Contacts-Controls Groups 3 and 4			*					*			*	

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

[B] Probability of TB group diagnosis by discriminant analysis. Analysis of the six parameters yields the highest discriminatory values of being a contact (84%) or becoming an acute (61%) or chronic (71%) TB patient, upon exposure to the microbe.

	Acute	Chronic	Contacts	Controls
12 parameters	0.67	0.71	0.76	0.62
6 parameters*	0.61	0.71	0.84	0.62
4 parameters**	0.61	0.53	0.79	0.62

* IFNγ-SER, IFNγ-PPD, sIL-2-SER, IL-10-NS, IL6-SER, IL-6-PPD.

** IFNγ-PPD, sIL2R-SER, IL-10-NS, IL-6-SER.

Figure 1. [A] IFNγ measurements in the serum (SER) and in supernatants from cells cultured without (NS) and with PPD (PPD). Values are presented as u/ml on a logarithmic scale. In all groups, cells stimulated with PPD yielded the highest amounts, followed by the spontaneous secretion in non-stimulated cells; the lowest levels were found in the serum. Below the bar graph is a table indicating significant differences between the groups. *P* values are denoted by stars. Patients tended to have higher levels of IFNγ in the serum than did contacts and controls, but contacts' cells showed the highest response to PPD. The other cytokine measurements are: **[B]** sIL-2R (pg/ml), **[C]** IL-10 (pg/ml), and **[D]** IL-6 (pg/ml).
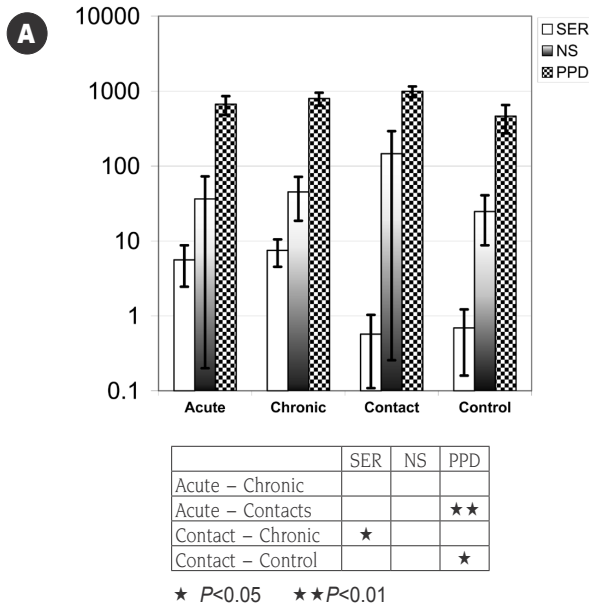
	SER	NS	PPD
Acute – Chronic			
Acute – Contacts			★★
Contact – Chronic	★		
Contact – Control			★

★ *P*<0.05   ★★*P*<0.01

Figure [A]. IFNγ profile (u/ml).
SER = serum, NS = unstimulated cells,
PPD = PPD stimulated cells

	SER	NS	PPD
Acute – Chronic		★	★★
Acute – Contacts	★★		
Contact – Chronic	★	★★	★
Contact – Control		★	
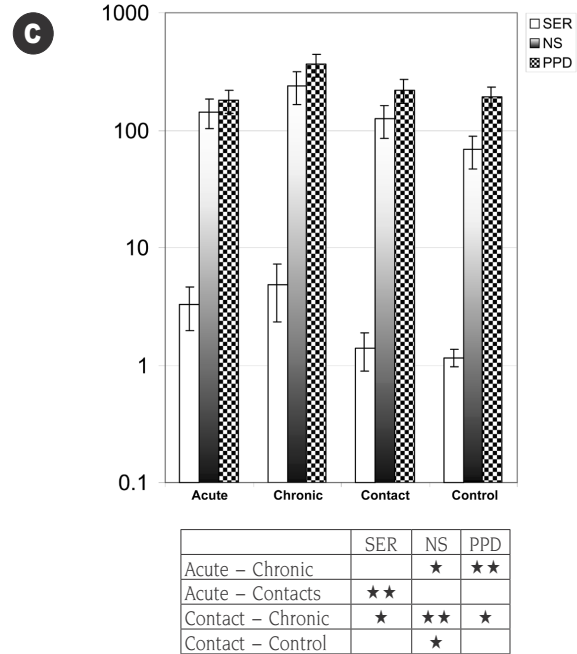
Figure [C]. IL-10 profile (pg/ml).

	SER	NS	PPD
Acute – Chronic	★★★		
Acute – Contacts	★★★	nd	
Contact – Chronic	★	nd	
Contact – Control		nd	
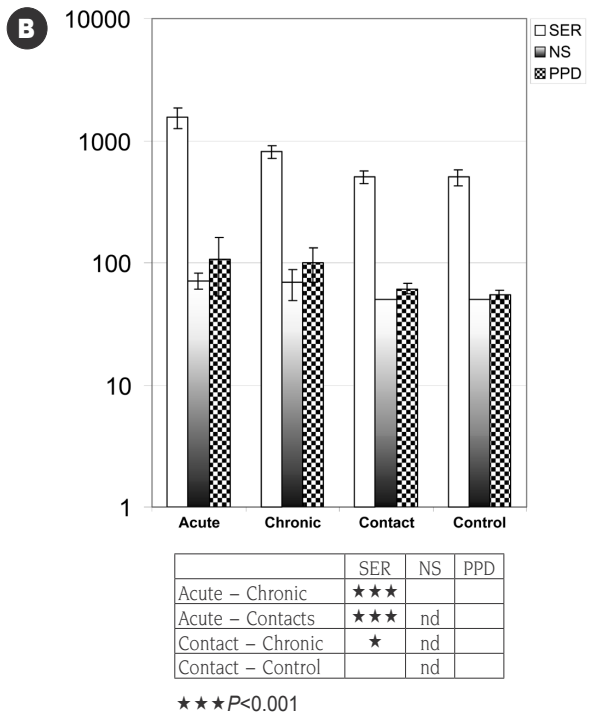
★★★*P*<0.001

Figure [B]. sIL-2R profile (pg/ml).
nd = not done

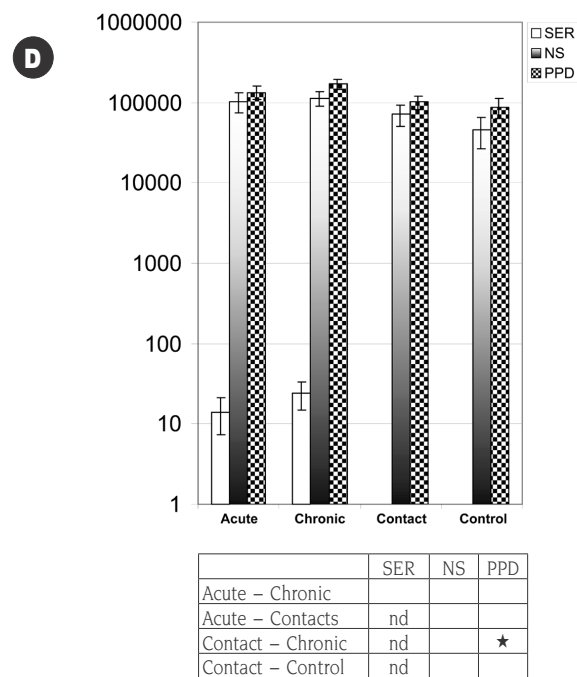|	SER	NS	PPD
Acute – Chronic			
Acute – Contacts	nd		
Contact – Chronic	nd		★
Contact – Control	nd		

Figure [D]. IL-6 (pg/ml).
nd = not done

observed between these two groups. PPD cells from patients in group 1 secreted more IFNγ than those in group 3 ($P > 0.01$) as well as more serum sIL-2R and IL-10 ($P > 0.001$ and 0.01, respectively). Group 3 had higher serum IFNγ, IL-2 and IL-10 ($P > 0.05$), and their non-stimulated cells as well as PPD cells secreted more IL-10 ($P > 0.01$ and 0.05, respectively), in comparison to group 2. The PPD cells of group 3 secreted more IFNγ, more non-stimulated cells and more IL-10 than those of group 4 ($P > 0.05$).

There was no difference in cytokine secretion between groups of TB patients and their contacts among Ethiopian families, as evaluated in part 2 of the study. However, when the family factor was introduced into the intergroup analysis, significant differences in IFNγ secretion among various families were revealed, irrespective of the family members being patients or contacts, and were not related to the clinical picture of the patients (F ratio = 1.98, probability level = 0.031). Other associations were not significant (data not presented).

Discussion

According to the current dogma regarding protective anti-mycobacterial host defenses, activation of CD4+ T cells by macrophages, and especially dendritic cells, are pivotal for an effective specific immune response, requiring mainly a Th1 type cytokine secretion, such as IFNγ and IL-12 [5,6]. Experimental models have shown that failure of this system results in rapid spread and multiplication of bacteria [6]. In humans, reduced *in vitro* production of IFNγ by PBMC in response to lectins and PPD was found in some patients [7].

Point mutations in IFNγ and IL-12 receptors [8,9], or the IL-12 signaling pathway [10], were found in cases of fatal or near-fatal disseminated infections with Bacille Calmette-Guérin or various mycobacteria. The latter findings, although important in themselves, were found in a limited number of families and cannot explain the spread of TB in apparently immunocompetent populations. Lately it has been claimed that the cause of the spread and virulence of the TB and human immunodeficiency virus epidemics in Africa is due to the high incidence of parasitic infections in general and of helminths in particular. Life in this milieu skews the cell-mediated response towards the Th2 type, as was observed in a group of Ethiopian immigrants in Israel [11].

However, we could not corroborate this hypothesis in a pilot study performed in Ethiopian children and adolescents with TB and TB contacts shortly after their arrival in Israel [4]. The patients with acute TB disease showed no delayed-type hypersensitivity anergy, their cells proliferated vigorously in response to PPD, and secreted mainly IL-2 and IL-6 and less IFNγ and IL-4. These results do not fit into a simplistic Th-1/Th-2 model of immune responses in TB.

Another study showed that patients and controls secreted IFNγ similarly, but increased IL-4 production was associated with pulmonary cavitary disease [12]. Another review summarizing the evidence indicated the need for the Th1 type response for the immediate containment of the infection and the Th2 type for late-phase granuloma formation [13]. Indeed, IL-6 knockout

mice had reduced IFNγ production, which was associated with an increased bacterial load [14].

The existence of checks and balances is further underscored by the finding that lack of IL-10, which down-regulates Th-1 responses [15] in knockout mice, leads to increased secretion of inflammatory cytokines and mediators and enhanced delayed hypersensitivity reactions [16]. Of equal importance for a successful anti-mycobacterial host response are chemokines and adhesion molecules, such as intracellular adhesion molecule-1, which also promote granuloma formation [17].

In the present study we assayed cytokine secretion in four groups of individuals, mainly of Ethiopian and East European background. These included patients with acute TB, chronic or relapsing TB, skin PPD-positive, asymptomatic TB contacts and PPD-negative controls, as well as Ethiopian families with one or more afflicted members.

Essentially, TB patients secreted significantly more of all the cytokines tested than the controls, and not less as might have been expected, without any Th type skewing. However, group 2, the persistently chronic or relapsing patients, showed a diminished response of their cells to PPD, in comparison to acute patients or contacts, but their levels were still higher than those of the controls who may represent the basal response of individuals not having been in contact with M. *tuberculosis*. At the same time, the chronic patients produced the highest levels of IL-10; this response may have contributed to the chronicity of their disease, although none of these patients was anergic. Furthermore, PPD-stimulated cells of contacts secreted more IFNγ and IL-2, which may have contributed to protection from overt disease. All the patients demonstrated a positive and increased Mantoux reaction. As in our previous study, we could not corroborate the presence of a specific immune impairment against TB antigens, or a generalized delayed-type hypersensitivity anergy. Also, no differences in the response were noted in correlation with a specific ethnic background.

However, a pattern emerged – namely, of the overall 12 immunological parameters tested, the combination of 6 of them gave the best prediction of remaining asymptomatic upon contact with TB (84%) as opposed to 61% to become a clinical TB patient. To the best of our knowledge, such an association has not been reported to date and it may explain a tendency towards mounting an effective immune response to TB, or not.

We have not attempted to correlate the present results with the administration of anti-tuberculous chemotherapy because the patients in our cohort were in various stages of treatment and a much larger series would be needed to divide into treatment subgroups. Immunomodulatory effects of anti-tuberculous drugs have been reported [18] and should be taken into consideration in future studies.

Nevertheless, it seems that the study of classical immunological parameters alone may be insufficient to explain the causes of the present global TB epidemic. Accordingly, it has been suggested that the failure of the immune anti-mycobacterial protection in developing countries is not due to a deficient Th-1 response, but rather its conversion into an immunopathologi-

cal one [19]. Possibly, a search for polymorphisms in candidate genes, such as IFNγ and IL-12 receptors, in large cohorts of people with a common genetic background, coupled with immune studies, may prove to be more fruitful. Indeed, a recent study has demonstrated a significant link between a 8q12-13 chromosomal locus and susceptibility to TB in a Moroccan cohort [20]. We are presently engaged in such a collaborative study in an Ethiopian immigrant cohort of Jewish descent.

The results of the second part of the present study, showing a family background-linked IFNγ response, may point towards this direction. Also, the enhanced IL-10 secretion by cells of the chronic patients, which may impede a rapid and effective Th-1 type anti-mycobacterial response, could have a genetic background.

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