Th2 biased immune response in cases with active 
*Mycobacterium tuberculosis* infection and tuberculin anergy

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Abstract

This study was aimed at investigating the immunologic relationship between cytokine production pattern and tuberculin negativity in patients with active *Mycobacterium tuberculosis* infection. After classifying patients by the extent of pulmonary involvement and the size of the tuberculin reaction, we evaluated the rate of cytokine positivity in peripheral blood to determine whether there is a characteristic cellular immune reaction pattern which could partly explain the tuberculin negativity in some of these cases. The significance of tuberculin anergy occurring in some cases with *M. tuberculosis* infection is still not clear. We investigated the ratio of IL-4, IL-10, IL-12, CD-4, CD-8 expressing lymphocytes in the peripheral blood of patients with active *M. tuberculosis* infection and correlated the percentage of the reactive cells with the positivity or negativity of tuberculin skin reactions. Twenty-eight patients were included in the study, with 11 healthy volunteers serving as controls. 10 ml of venous blood was drawn before starting anti-mycobacterial treatment. A tuberculin skin test was performed, introducing intracutaneously 5 TU PPD on the forearm with results evaluated after 72 h. Consistent with the reactivity or non-reactivity of the tuberculin skin test, we found a significantly higher ratio of IL-4 and IL-10 positive lymphocytes and a significantly lower ratio of IL-12 in the peripheral blood of patients with tuberculin anergy than in that of tuberculin positive patients or healthy donors. There was no difference in the ratio of the CD-4 CD-8 positive lymphocytes among the three groups. To evaluate whether the differences could be explained by the degree of pulmonary tubercular involvement, we classified the patients into three groups according to the extent and type of X-ray findings. Seven out of eight tuberculin negative patients were classified as grade III, whereas in the tuberculin positive group only seven out of 20 fell in this category. There was no significant correlation between the radiological grade of the patients and the examined in vitro parameters unless the tuberculin reactivity of each patients was also considered. Tuberculin anergy may reflect an inappropriate immune response to the intracellular pathogen. The high percentage of IL-4 and IL-10 positive lymphocytes together with a low percentage of IL-12 positive lymphocytes in the peripheral blood of anergic patients suggests a Th2 biased immune response during the early course of the disease.

Keywords: Interleukin-4; Interleukin-10; Interleukin-12; Mycobacterium tuberculosis; Tuberculin anergy

1. Introduction

Nearly 1 billion 700 million individuals throughout the world are infected with *Mycobacterium tubercu*...
and thus are carriers of live bacilli able to colonize and disseminate [1]. In the era of multidrug resistant bacilli, besides producing more effective antibiotics, evaluation of the immunologic events during M. tuberculosis infection may be important for the development of additional therapeutic approaches to combat this potentially life-threatening disease.

Murine CD4+ T cells have been divided into at least two different subsets (Th1 and Th2), based on the cytokine profiles that they secrete upon antigenic stimulation. Th1 cells characteristically secrete interleukin-2 (IL-2) and interferon-gamma (IFN\(\gamma\)), whereas Th2 lymphocytes typically produce IL-4, IL-5, and IL-10, which enhance antibody synthesis of B cells and play a role in allergic diseases. CD4+ T cells with an intermediate cytokine profile (Th0) have also been described [2]. Mycobacteria preferentially induce Th1-like responses, as reflected by the production of high titers of IFN\(\gamma\) and TNF\(\alpha\) with low or undetectable levels of IL-4 [3]. IL-12 favors the development of Th-1-like T cell responses by enhancing IFN\(\gamma\) and antagonizing IL-4 and IL-10, thereby down-regulating the Th-2 responses [4]. Patients with tuberculosis infection frequently have a depressed cellular and an increased humoral immune response against mycobacterial antigens [5]. IL-4 and IL-10 are known to inhibit significantly the development of delayed type hypersensitivity (DTH) responses in mice [6]; IL-10 inhibits Th1 activity through macrophage deactivation and the blocking of IFN\(\gamma\) release by Th1 lymphocytes [7]. IL-12 is a key cytokine in immune regulation, presumably by inducing commitment from T helper 0 (Th0) to the Th1 phenotype [8], and is held to be a marker of active disease in pulmonary tuberculosis [9]. Consequently, we examined the ratio of the IL-4, IL-10, and IL-12 positive lymphocytes as well as that of CD-4 and CD-8 positive lymphocytes in the peripheral blood of our patients with active M. tuberculosis infection in the context of their DTH reaction.

Patients with tuberculin anergy usually have more advanced disease than those with a positive tuberculin reaction. Therefore, it was also necessary to look at whether the severity of pulmonary involvement could be consistently related to the presence of tuberculin anergy and/or any resulting immunologic response.

1.1. Aims of the study

This study was aimed at investigating the immunologic relationship between cytokine production pattern and tuberculin negativity in patients with active M. tuberculosis infection. After classifying patients according to the extent of pulmonary involvement and the size of the tuberculin reaction, we evaluated the rate of cytokine positivity in peripheral blood to determine whether there is a characteristic cellular immune reaction pattern which could partly explain the tuberculin negativity in some of these cases.

2. Materials and methods

2.1. Subjects

Twenty-eight patients with M. tuberculosis were included in the study (Table 1). All of them had a positive sputum culture for M. tuberculosis and 15 patients showed smear positivity. The patients were checked for HIV infection; all were negative (the incidence of HIV infection in Hungary is still very low). A tuberculin skin test as part of the clinical evaluation of the patients was performed by introducing 5 TU PPD (Human, Hungary) intracutaneously on the forearm, and results were evaluated after 72 h. All skin tests were administered by the same individual and were read blind to the diagnosis of the patient and to the result of the lymphocyte analysis. The skin test was considered positive if there was induration \(\geq 10\) mm and negative if there was no reaction (among our patients there were no cases with reactions between 1 and 10 mm).

Patients were classified according to the extent and type of X-ray findings into three groups following the classification of Dlugovitzky et al. [10]: group 1 = grade I, mild cases, \((n = 8)\), patients with a single lobe involved and without visible cavities; group 2 = grade II, moderate cases \((n = 6)\), patients presenting unilateral involvement of two or more lobes with cavities, if present, reaching a total diameter no greater than 4 cm; and group 3 = grade III, advanced cases \((n = 14)\), bilateral disease with massive involvement and multiple cavities (Table 2).

Eleven healthy volunteers (10 female, one male)
serving as controls were all nurses in our department and were therefore in contact with tuberculotic patients; none, however, had a history of tuberculosis but all had been vaccinated with BCG in childhood. Permission for the study was obtained from the Clinical Ethics Committee of the Medical University of Pécs, and all subjects gave their consent to participate.

2.2. Isolation of lymphocytes

Ten ml of venous blood was drawn before initiating anti-mycobacterial treatment. Lymphocytes were separated from heparinized venous blood on the Ficoll-Paque (Pharmacia) gradient. The cells were washed in RPMI 1640 medium (Gibco) and centrifuged on microscope slides at a cell count of $1 \times 10^6$ ml$^{-1}$. The purity of the isolated population was periodically checked for reactivity to anti-CD3 antibody and was found to be consistent.

2.3. Identification of IL-4, IL-10, IL-12, CD-8, and CD-4 positive lymphocytes

IL-4, 10, and 12 are intracellular antigens secreted on the cell surface. The cells were fixed for 5 min in cold acetone. Acetone permeabilizes the cell membrane and allows IgG to penetrate the membrane. Following acetone treatment the cells were reacted with polyclonal antihuman anti-IL-10 antibody (R&D Systems) (both diluted 1:500) for 60 min at room temperature in a humid atmosphere. As a second antibody we used peroxidase labeled anti-goat IgG (Dako) (1:100). In the case of IL-12 we used anti-IL-12 monoclonal antibody (R&D Systems) at a dilution of 1:100 and as a second antibody we used peroxidase labeled anti-mouse IgG (Dako) (1:100). To identify CD8 and CD4 positive lymphocytes we used mouse monoclonal anti-human CD8 antibody (Becton Dickinson) and monoclonal anti-human CD4 antibody (Becton Dickinson) diluted 1:50 and as a second antibody we used peroxidase labeled anti-mouse IgG (Dako) (1:100). Reactions were developed by diaminobenzidine, intensified with silver staining. The percentage of IL-4, IL-10, IL-12, CD-4, and CD-8 positive T cells was determined by microscopy counting 300 lymphocytes at high power magnification. The slides were coded and assessed by one individual.

2.4. Statistics

The two-tailed Student’s $t$-test was used in our statistical analysis of the data.

3. Results

Patients were classified according to the extent and type of X-ray findings into three groups follow-

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td>Classification of the radiologic manifestation of patients with pulmonary tuberculosis and tuberculin skin test reactivity</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Patients</td>
</tr>
<tr>
<td>Tuberculin skin test</td>
</tr>
<tr>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
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</table>
ing the classification of Dlugovitzky et al. [10] (Table 2).

The ratio of IL-4, IL-10, IL-12, CD-8, and CD-4 positive lymphocytes was determined in peripheral blood of tuberculosis patients and data were analyzed in the context of tuberculin reactivity. Because the control group was recruited exclusively from female volunteers, there were no differences between the sexes in the ratio of IL-4, IL-10, IL-12, CD-8, and CD-4 positive lymphocytes. The percentage of IL-4 positive cells was significantly higher in patients with negative tuberculin reactions than in those with positive tuberculin reactions or in healthy volunteers (Table 3). Similar results were obtained for IL-10 (Table 3). In contrast, the ratio of IL-12 positive cells was significantly lower in patients with negative tuberculin reactions than in those with positive tuberculin reactions or healthy volunteers (Table 3). There was no significant difference between the ratio of CD-8 and CD4 positive lymphocytes in the peripheral blood of patients with either anergic or positive tuberculin reactions and of the healthy volunteers (Table 3).

Following classification of patients into three groups according to the extent and type of X-ray findings, we found that among the eight tuberculin anergic cases, seven were in group 3 (patients with advanced pulmonary manifestation of the disease). Among 19 patients with positive tuberculin skin reaction, seven were also in group 3; six belonged to group 2, and six to group 1. Cytokine expression on the lymphocytes showed no relationship with X-ray findings.

4. Discussion

It is known that mycobacteria predominantly induce CD4+ Th1 cells and CD8+ cytotoxic T cells with a Th1-like cytokine profile of elevated IL-2 and IFNγ levels [3]. Recently it was found that the percentage of IL-12 positive cells is more than three times that of the IFNγ positive cells in patients with active tuberculosis [9]. The presence of a Th2 response or of type 2 cytokines IL-4 and IL-10 is associated with progressive disease [11], whereas a Th1-type response has been linked to protective immunity. IL-4, IL-10, IL-13, and transforming growth factor beta comprise the quartet of defined macrophage deactivating cytokines [12]. IL-4 blocks IL-2 and IFNγ secretion by polyclonally stimulated human T cells [13] and has a selective potentiating effect on the proliferation and cytokine synthesis of Th2 clones, while IL-10 might be involved in damping ongoing antigen driven immune responses rather than in the selective regulation of Th1 functions [14]. Thus IL-4 and IL-10 could be associated with diminished resistance to infection by mycobacteria [15].

Evaluating patients with active M. tuberculosis infection, we found that patients with tuberculin anergy had a significantly higher ratio of IL-4 and IL-10 positive lymphocytes in peripheral blood than either those with a positive tuberculin skin test or healthy volunteers. On the other hand, patients with tuberculin anergy had a significantly lower ratio of IL-12 positive lymphocytes than either those with a positive tuberculin skin test or healthy volunteers.

Table 3
Percentage of lymphocytes positive for markers and cytokines in the peripheral blood of patients with active M. tuberculosis in relation to their tuberculin skin test reaction

<table>
<thead>
<tr>
<th>Tuberculin skin test</th>
<th>Percentage of positive lymphocytes (± S.E.M.) in the peripheral blood reaction</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IL-4</td>
</tr>
<tr>
<td>Positive</td>
<td>5.76 ± 1.36</td>
</tr>
<tr>
<td>n = 13</td>
<td>n = 12</td>
</tr>
<tr>
<td>Negative</td>
<td>36.14 ± 7.72*</td>
</tr>
<tr>
<td>n = 7</td>
<td>n = 4</td>
</tr>
<tr>
<td>Healthy controls (n = 11)</td>
<td>5.72 ± 1.48</td>
</tr>
</tbody>
</table>

*Significant difference between tuberculin negative and tuberculin positive cases (P < 0.01) and significant difference between tuberculin negative cases and healthy controls (P < 0.01). **Significant difference between tuberculin negative and tuberculin positive cases (P < 0.02) and significant difference between tuberculin negative cases and healthy controls (P < 0.02). NS, not significant.
There were no differences in the ratio of CD-8 and CD-4 positive lymphocytes.

As noted, patients with tuberculin anergy usually have more advanced disease than patients with a positive tuberculin reaction; therefore, it was important to evaluate whether the results simply correlate with the degree of pulmonary involvement of the disease or the tuberculin reaction has its own impact on the result independently. Seven out of eight tuberculin negative patients were classified as grade III whereas in the tuberculin positive group only seven out of 20 fell in this category. We found that there was no significant correlation between the radiologic grade of the patients and the in vitro immunologic parameters examined unless the tuberculin reactivity of each patient was also considered. Based on these findings, we suggest that the cytokine pattern correlates with the degree of the tuberculin skin test and consequently with the pulmonary manifestation of the disease.

We suggest that the increased ratio of IL-4 and IL-10 positive lymphocytes and the decreased ratio of IL-12 positive lymphocytes contributes to the tuberculin skin anergy and, in part, also for the progression of the disease. It should be emphasized that among our patient groups, only four were over 60 years old; thus we believe that the age of those studied had no effect on the observed tuberculin anergy.

It has been reported previously that there is a significant difference in the production of IL-4 among tubercular patients and healthy controls, as most patients exhibit a Th2 pattern of immune responsiveness whereas tuberculin positive healthy individuals have a Th1 pattern in vitro [5]. A similar observation was made by Surcel et al., who concluded that IL-4 production may be involved in the loss of protective host response and thereby be linked to the pathogenesis of tuberculosis [2]. If true, it is tempting to speculate that, as in experimental mouse models [16], anti-IL-4 and anti-IL-10 therapies might be used in the treatment of selected cases with advanced disease, particularly in those infected with multi-drug resistant bacilli. It is possible that an antagonist of disease promoting cytokines might be useful in enhancing host resistance following immunologic manipulations which eliminate the Th2 component and boost the type 1 response [16,17].

5. Conclusions

Our results indicate that in patients with active *M. tuberculosis* infection who are tuberculin negative there is a Th2 biased immune response which could explain both the advanced pulmonary involvement and the anergic skin reaction in these cases.

References


