Cellular and Humoral Immunity to Purified Protein Derivative (PPD) in PPD Skin Reactive and Nonreactive Patients with Pulmonary Tuberculosis: Comparative Analysis of Antigen-specific Lymphocyte Proliferation and IgG Antibodies

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Aim. To evaluate in vitro reactivity against tuberculin purified protein derivative (PPD) in patients with active pulmonary tuberculosis scoring either positive or negative upon intradermal PPD application (PPD-DTH).

Method. Two groups of patients with pulmonary tuberculosis, 22 PPD+/c43 and 22 PPD+/c45, were studied. Peripheral blood mononuclear cells (PBMC) were assayed for in vitro proliferation to PPD antigen, phytohaemagglutinin, concanavalin A, and pokeweed mitogens. In the proliferation assay PBMC were incubated in a medium supplemented with serum (20% concentration) from healthy donors, autologous serum, or allogenic serum. Anti-PPD IgG concentration in patients’ sera were analyzed by ELISA. CD3+ lymphocytes from 10 patients in each group were tested for the expression of surface activation markers (HLA-DR and CD25/IL-2 receptors) by flow cytometry.

Results. PPD+/c45 patients showed clinically and radiologically more advanced forms of pulmonary tuberculosis as compared with PPD+/c43 patients. PBMC from both groups of patients proliferated in response to PPD effectively, but significantly higher de novo DNA synthesis was observed in PPD+/c43 patients (p<0.001). Proliferative activity was not affected by the type of the serum supplement (autologous or allogenic) in the culture medium. Mitogen stimulation elicited similar proliferative responses in both groups. Similar percentages of T-lymphocytes and T-lymphocytes expressing CD25 proliferation markers were observed in both groups of patients. There was a borderline difference in the percentage of CD3+HLA-DR+ lymphocytes between these two groups of patients (p=0.05). At 1:1000 serum dilution a significant difference (p=0.002) in anti-PPD IgG concentrations was found between PPD+/c43 and PPD+/c45 patients.

Conclusion. Patients with active pulmonary tuberculosis with a more favorable clinical course have a more potent specific cell-mediated immunity to PPD (positive skin reactivity in vivo and significantly greater lymphocyte proliferative response in vitro) than patients with a clinically more severe form of the disease. The concentration of PPD specific IgG in the serum appears to be higher in patients with relatively more severe forms of the disease.

Key words: antibody specificity; IgG; immunity; T-lymphocytes; tuberculin test; tuberculosis, pulmonary

The contact with Mycobacterium tuberculosis raises cellular and partly humoral immune responses (1-4). The formation of characteristic localized inflammatory cellular immune response, ie, granulomas, is critical to the resolution and control of infection. Granulomas are composed of activated mononuclear phagocytic cells and T lymphocytes. The evolution of the granuloma cellular composition during infection is less well defined (5-9). The formation of granulomas is dependent on cytokines, such as interleukin 2 (IL-2) (6-7), gamma interferon (IFN-γ) (6,10), and particularly on tumor necrosis factor (TNF) (11) and lymphotokin alpha (LT-α) (12). The role of humoral response in the immunopathogenesis of tuberculosis has yet to be fully elucidated (13-16).

Active tuberculosis usually provokes a strong hypersensitivity to mycobacterial antigens, which can be evaluated by tuberculin purified protein derivative (PPD) skin test. However, there are active tuberculosis patients who are unreactive to PPD skin test (9,17-19), a state clinically defined as “anergy”. Such patients usually show impaired delayed-type hypersensitivity (DTH) response to specific antigens, in the absence of generalized state of immunosuppression. It has been suggested that this specific anergy, initially ascribed to poor nutrition of the patient and severity of the disease, may be caused by an adherent suppressor monocyte population (2,20-22).

The division of clinical course of tuberculosis in two phases according to PPD-reactive and nonreactive phases of the disease has been proposed. The reactive phase would be marked by strong cellular response, low antibody titers, and few bacilli, whereas in nonreactive or anergic patients cellular response...
might be weaker or absent, titers of antibodies high, and bacilli present in large numbers (14,16). Remarkably, specific antibodies or immune complexes have been held responsible for the inhibition of lymphocyte proliferation in anergic tuberculosis patients (23).

We evaluated cellular and humoral immunity in patients with active tuberculosis, classified according to their skin reactivity to PPD in the absence of other potentially immunocompromising diseases or therapies. We found a certain parallelism between DTH reactivity to PPD and anti-PPD proliferative capacity of lymphocytes in vitro, as well as a difference in the specific humoral response between the two groups of patients.

**Patients and Methods**

**Patients**

The study included unrelated patients with pulmonary tuberculosis admitted to a specialized institution (Hospital for Lung Diseases and Tuberculosis, Klenovnik, Croatia). The diagnosis of active tuberculosis was based on typical clinical, laboratory, radiological, and microbiological findings (2,3). Patients suffering from concomitant diseases, patients undergoing medical treatments with possible immunosuppressive effects, and pregnant patients were not included in the study. Based on their skin reactivity to PPD (Mantoux test, see below), the patients were divided into PPD-nonreactive (PPD−) and PPD-reactive patients (PPD+). Twenty-two patients of both sexes were randomly selected from each of the two groups, reaching the total number of 44. Patients' age ranged between 25 and 58 years. Except in body weight, the two groups were comparable in their basic anthropometric parameters (Table 1). The Ethics Committee of the Hospital approved the study protocol.

Table 1. Characteristics of PPD+ and PPD− patients with pulmonary tuberculosis according to age, sex, body weight, height and the skin size of PPD-DTH reaction

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PPD+ (n=22)</th>
<th>PPD− (n=22)</th>
<th>z</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>38 (25-56)</td>
<td>41 (29-57)</td>
<td>-4.943</td>
<td>0.046</td>
</tr>
<tr>
<td>No. of men</td>
<td>17</td>
<td>20</td>
<td>1.529</td>
<td>0.216</td>
</tr>
<tr>
<td>No. of women</td>
<td>5</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>68 (57-98)</td>
<td>63 (41-74)</td>
<td>-2.221</td>
<td>0.026</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>172 (164-195)</td>
<td>172 (160-183)</td>
<td>-0.409</td>
<td>0.682</td>
</tr>
<tr>
<td>PPD-DTH reaction (mm)</td>
<td>15 (10-30)</td>
<td>negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mann-Whitney U-test.

**Tuberculin Skin Test**

Skin tests were performed according to Mantoux, with 0.1 ml volumes of PPD stabilized with Tween 80 (PPD RT 23, Croatian National Institute of Public Health, Zagreb, Croatia). Three tuberculin units (TU) were injected intradermally into the upper third of patients' heparinized peripheral blood by centrifugation over a Ficoll layer (Lymphoprep, Nycomed, Oslo, Norway d=1.077 g/ml) followed by two washes in phosphate-buffered saline (PBS). Suspensions of peripheral blood mononuclear cells were incubated for 30 min at +4°C in the dark, in the presence of fluorescein isothiocyanate (FITC)-labeled anti-CD3 specific monoclonal antibodies, together with phycoerythrin (PE) CD25- (IL-2R) or HLA-DR-specific monoclonal antibodies or isotypic controls (Coulter Mouse IgG1, IgG2b, and IgG2a). After incubation, washings, and resuspension, the fluorescence of cells was analyzed on a flow cytometer (Coulter, Hialeah, FL, USA; Argon laser 488 nm, 250 mW). Ten thousand cells were counted in each sample and lymphocyte gates were set by use of forward angle vs right angle light scatter parameters. The controls with monoclonal antibodies CD45-FITC/CD14-PE confirmed that 95% or more of gated cells were of lymphocyte origin. By means of red and green fluorescence of stained lymphocytes in a sample, the percentages of cells bearing only one, both (CD3 + CD25 or CD3 + CD25 + HLA-DR), or neither of the cell-surface antigens were calculated (27). Ten patients from each group were studied.

**Lymphocyte Proliferation Assays**

Lymphocyte proliferation assays were done by testing concomitantly one PPD+ patient and one PPD− patient. This allowed us to use their sera for cell culture medium supplementation in terms of autologous or allogenic sera. Peripheral blood mononuclear cells were resuspended in M199 medium supplemented with 20% heat-inactivated autologous sera and pooled human AB serum, as indicated. Triplicate cultures containing 10^5 cells/0.25 ml were plated in 96 well flat-bottomed (Falcon, Becton Dickinson Labware, Becton Dickinson Company, Lincoln Park, NJ, USA). Phytohemagglutinin (PHA, 10 μg/ml), Concanavalin A (Con A, 20 μg/ml) and Pokeweed mitogen (PWM, 10 μg/ml) from Sigma (St. Louis, MO, USA) and tuberculin purified protein derivative (PPD, Statens Seruminstitut, Copenhagen, Denmark, 5 and 10 μg/ml) were used as stimulating agents. After five days, 1 μCi of [3H]-TdR (Amersham, Buckingham, UK) was added to each well and cultures were harvested 18 h later (9,28). Lymphocyte proliferation was measured as mean counts per minute (cpm) and results were expressed as stimulation indices (SI) calculated by dividing experimental [3H] incorporation by those obtained in unstimulated cultures. SI >3 were considered positive (28,29).

**Determination of PPD-specific IgG in Sera**

High absorption ELISA plates (Falcon) were coated with PPD antigen (50 μg/ml dissolved in bicarbonate buffer, 100 μl/well) and incubated overnight at +4°C. Following washes and saturation of unbound sites, patients sera (100 μl/well) were added at different dilutions and incubated at room temperature for an hour. Specific binding was revealed by horseradish peroxidase (HRP)-conjugated secondary antibody upon reading absorption at 492 nm wavelength (30).

**Statistical Analysis**

Data were statistically analyzed by Student’s t-test, Chi-square test, Fisher’s exact probability test, and Mann Whitney U-non-parametric test for two independent samples (SPSS Inc, Chicago, IL, USA) (31).

**Results**

Both groups of patients showed the same percentage of Mycobacterium tuberculosis positive material (Table 2). PPD+ patients generally displayed extensive bilateral radiological lung alterations consistent with active tuberculosis, unlike PPD− patients whose radiological lung alterations were less pronounced. Also, most PPD− patients had a recurrence of tuberculosis, whereas PPD+ patients were mostly newly affected patients.

Flow cytometry analysis of T-lymphocytes (CD3+ and CD3HLA-DR and CD3CD25 lymphocytes was performed on peripheral blood mononuclear cells from randomly selected 10 PPD+ and 10 PPD− patients (Table 3). The PPD− and PPD+ patients did not significantly differ in the number of T-lymphocytes (CD3+ lymphocytes) and their surface expression of CD25 activation markers, which were also within the limits of the reference values observed in healthy donors. There was a borderline significance in the per-
Proliferative responses to mitogenic stimuli in the two groups were not significantly different, irrespective of the type of serum supplement (pooled AB, autologous or allogenic) to the culture medium (Table 4). On the other hand, PPD specific response in vitro was significantly higher in patients with positive than in those with negative skin test. Remarkably, responsiveness to PPD in skin-test-positive patients did not appear to be inhibited in cultures performed in the presence of sera from skin-test-negative patients (Table 4). Reciprocally, the type of serum supplement did not affect the specific PPD-hyposensitivity of the skin-test-negative patients.

PPD-specific IgG were detected in both skin-test-positive and negative patients. A trend towards higher values of PPD specific IgG was observed in the PPD+ patients and these values reached the level of statistical significance at dilution of sera 1:1000 (Table 5).

Discussion

PPD anergy in tuberculosis patients reflects weak or absent cellular reactivity, the underlying molecular background of which is still unclear (7-9). We have analyzed cellular and humoral immunity in patients with active pulmonary tuberculosis, classified according to their PPD skin reactivity. We found a correlation between DTH reactivity to PPD and anti-PPD proliferative capacity of lymphocytes in vitro and differences in specific humoral responses between the two groups of patients.

Lenzini and coworkers (14) suggested a correlation between PPD skin reactivity and forms of tuberculosis with local lesions, with anergy occurring in forms characterized by extensive lung disease. Common feature of anergic patients is their unresponsiveness to drug therapy and the development of recurrent, severe tuberculosis (23). In our study many of PPD patients suffered an extensive recurrence of the disease (Table 2), which is a finding consistent with previous reports (23).

Shingal et al (32) found reduced number of CD4+ cells and increased number of CD8+ cells in patients with active tuberculosis, which normalized after three months of antituberculosis therapy. Mishin et al (33) found a reduced total T cell number in such patients, with altered functions of immunocompetent cells, Rossi and coworkers (34) suggested that early skin anergy in patients with tuberculous effusions...
may be connected with sequestration of PPD reactive T-lymphocytes in the pleural spaces. More recently, Condos et al (35) found IFN-γ but not IL-4 secretion in the bronchoalveolar lavage in patients with mild clinical forms of tuberculosis. At mRNA level, increased IL-12 and IFN-γ gene transcription was observed in bronchoalveolar lavage samples of patients with active pulmonary tuberculosis, as opposed to the patients with inactive form, whereas no significant differences in IL-2, TNF-α, IL-4, and IL-5 gene expression were found between these two groups of patients (36).

The precise role of IL-10, an immunoregulatory cytokine that inhibits both Th1-like T cell responses and macrophage activation, is still not clear (37). Results from experimental animal studies suggested that IL-10 knockout mice (IL-10−/−) might be more resistant to mycobacterial pathogens, i.e., they had a better clearance of mycobacterial organisms, although it varied depending on the mycobacterial species (38). For example, endogenous IL-10 inhibits significantly specific IFN-γ T cell responses to M. avium infection. However, during M. tuberculosis infection this effect is short-lived and fails to influence the long-term course of infection (37). On the other hand, in vitro stimulation of T cells with PPD induced cell proliferation and production of IL-10 and IFN-γ in PPD+ patients, whereas in anergic patients it induced only IL-10 production but not IFN-γ or proliferation (9). We found that the total number of T-lymphocytes and their expression of activation markers were similar in PPD− and PPD+ patients.

It has been suggested that unresponsiveness to PPD in PPD+ patients might be caused by inhibitory substances present in their sera (13,23). Therefore, we investigated in vitro proliferation of lymphocytes in different culture conditions: in the presence of autologous serum, serum from pooled AB+ healthy donors, or allogenic serum from PPD+ or PPD− patients. Lymphocytes from a PPD+ patient were incubated in the medium with the serum from the PPD− patient and vice versa. PPD and PHA, Con A, and PWM mitogens were used as stimuli. Response to mitogens was similar in both groups of patients, whereas proliferation to PPD was virtually absent in skin-test-negative as opposed to skin-test-positive patients, whereas proliferation to PPD was virtually absent in skin-test-negative as opposed to skin-test-positive patients.

References
36 Taha RA, Kotimbos TC, Song YL, Menzies D, Hamid Q. IFN-gamma and IL-12 are increased in active compared with inactive tuberculosis. Am J Respir Crit Care Med 1997;155:1135-9.

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