Lymphocyte Proliferation and Gamma-Interferon Production after "in Vitro" Stimulation with PPD*

Differences between Tuberculous and Nontuberculous Pleurisy in Patients with Positive Tuberculin Skin Test

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T-lymphocytes previously sensitized by an antigen undergo blastic transformation and produce IFNγ when stimulated by the same antigen. We studied the lymphoblastic response to PPD and IFNγ production in pleural fluid and peripheral blood of 41 patients (15 with tuberculous pleural effusion, 13 with nontuberculous pleurisy and positive tuberculin skin test, and 13 with tuberculin-negative nontuberculous pleurisy). In tuberculous pleuritis, pleural lymphocyte blastic response and IFNγ production were higher than those of peripheral lymphocytes, whereas in tuberculin-positive nontuberculous patients, peripheral lymphocyte response and IFNγ production were higher than those of pleural lymphocytes. Tuberculous pleural fluid lymphocytes underwent greater blastic transformation and produced more IFNγ than pleural lymphocytes of tuberculin-positive nontuberculous patients, whereas the opposite occurred in peripheral lymphocytes. In tuberculin-negative nontuberculous patients, there was no lymphoblastic response in either the pleural fluid or peripheral blood. These results concur with the concept of immunologic compartmentalization. In tuberculous pleuritis, there would be clonal expansion of PPD-responding T-lymphocytes in the pleural compartment. This expansion of PPD-specific lymphocytes would not occur in nontuberculous pleuritis, but lymphocytes sensitized to other antigens would accumulate in the pleural compartment.

(Chest 1990; 97:1381-85)

IFNγ = interferon gamma; ADA = adenosine deaminase; RIA = radioimmunoassay

T-lymphocytes, mainly the CD4+ subpopulation, predominate in lymphocytic pleural fluid of different etiologies, which suggests that cellular immunity plays an important role in the pathogenesis of the disease. Gamma-interferon is produced by sensitized lymphocytes stimulated by the same antigen. Some studies show that lymphocytes in patients with tuberculosis undergo blastic transformation when stimulated with PPD and produce IFNγ; the response of pleural lymphocytes is greater than that of peripheral lymphocytes. There is almost no mention in the literature of the lymphocytic response to PPD in nontuberculous pleuritis in patients with a positive cutaneous tuberculin reaction, in whom lymphocytes would also be sensitized by previous contact with tuberculous antigens.

The blastic response to PPD and IFNγ production in pleural fluid and peripheral blood of patients with lymphocytic pleural effusion were studied simultaneously, with special reference to differences between tuberculous and nontuberculous tuberculin-positive patients.

Materials and Methods

Patients

We studied 41 of our hospital inpatients with lymphocytic pleural effusion. According to their final diagnosis, these patients were classified into three groups:

- Group 1 was 15 patients with tuberculous pleural effusion. Diagnosis was confirmed by bacteriologic or histopathologic studies or both. The tuberculin skin test with 5 IU of PPD was positive (>10-mm diameter of skin induration) in 13 cases and negative in two. In all cases, the studies were carried out before antituberculosis therapy was started.
- Group 2 consisted of 19 patients with malignant pleural effusion. Diagnosis was confirmed by histologic or cytologic studies or both. The tuberculin test was positive in ten cases and negative in nine. In all cases, studies were performed before chemotherapy was started.
- Group 3 was seven patients with pleural effusion of unknown origin. In all cases, pleural biopsy showed nonspecific pleuritis. The pleural fluid was inflammatory with predominance of lymphocytes, ADA activity was lower than 20 units/ml, and the clinical course was favorable, with spontaneous resolution of the effusion without specific therapy. The tuberculin test was positive in three cases and negative in four.

According to the results of the tuberculin skin test, patients from...
groups 2 and 3 (nontuberculous pleurisy) were regrouped as follows: group 2, nontuberculous tuberculin-positive pleurisy (13 cases); and group 3, nontuberculous tuberculin-negative pleurisy (13 cases).

**Lymphocyte Preparation and Culture**

The PPD was supplied by Statens Seruminstitut (Copenhagen) and was used at a dilution of 25 μg/ml of RPMI 1640 (Flow Lab) with 10 percent fetal calf serum.

Pleural fluid (obtained by thoracentesis) and peripheral venous blood were collected on the same day in sterile tubes containing heparin. Mononuclear cells were separated by Ficoll-Hypaque gradient (Pharmacia Fine Chemicals). After centrifugation at 400 g for 30 minutes, the mononuclear cell interphase was obtained, was washed twice in Hanks' saline solution (Gibco), and was resuspended in culture media. Mononuclear cells were counted in a hemacytometer, and the cell concentration was adjusted to 2.5 × 10^6 cells per milliliter, to which PPD was added at the doses indicated, and antigen-free cultures were left as controls. Cultures were then incubated for five days in a 5 percent CO₂ atmosphere at 37°C. All cultures were made in duplicate (results are expressed as mean values).

**Lymphocyte Blastogenesis Assay**

Lymphocyte transformation was measured by the incorporation of 0.5μCi of tritiated thymidine (TRK-120, Amersham) to lymphocyte DNA during the last 18 hours of incubation. Supernatants were then aspirated and cells lysed with 5 percent acetic acid. The contents of each tube were harvested onto glass-fiber disks for scintillation counting. The radioactivity recovered from the disks was measured in a liquid scintillation β-counter. The lymphocyte proliferation index is the ratio between the counts per minute obtained from PPD-stimulated culture and the counts per minute from the nonstimulated control culture. The five-day culture and the concentration of PPD described previously yielded optimal responses in this assay.

**Interferon Gamma Assay**

The IFNγ was assayed in supernatants from the aforementioned cultures by a solid-phase RIA, based on the "forward sandwich" principle. The RIA kits (IMRX Interferon-Gamma RIA Centocor) contained antibody-coated polystyrene beads and a ^125I-labeled solution of a second antibody. A polystyrene bead, coated first with murine monoclonal antibody specific for human IFNγ, was incubated for two hours in 200μl of either the test culture supernatant or the vendor-supplied standard or control supernatant. During this incubation, IFNγ in the specimen was bound to the solid phase. The bead was washed twice to remove unbound material. A second murine antibody to human IFNγ labeled with ^125I was incubated for two hours with the bead, and unbound labeled antibody was removed by washing the beads twice. The bound radioactivity was then determined by counting the beads in a gamma scintillation counter. Bound radioactivity was directly proportional to the concentration of IFNγ present in the specimen. Results are measured in units per milliliter (reference on the value of National Institutes of Health standard). This assay is sensitive (lower limit, 0.1 units/ml) and specific (the RIA does not detect IFNα or IFNβ). The IFNγ stimulation index was defined as the ratio of the units per milliliter of IFNγ in the supernatant of the culture stimulated by PPD to the units per milliliter of IFNγ in the supernatant of the

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<th>NON-TBC PPD+</th>
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<td>18</td>
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<td>18</td>
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<tr>
<td>P. FLUID</td>
<td>p &lt; .001</td>
<td>p &lt; .005</td>
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**FIGURE 1.** PPD-stimulated lymphocyte proliferation index in pleural fluid and blood of patients with tuberculous pleural effusion (I), nontuberculous tuberculin-positive pleurisy (II), and nontuberculous tuberculin-negative pleurisy (III). In group I, open circles indicate tuberculin-positive patients.
control culture (without PPD).

Statistical Analysis

Statistical analysis was performed using the SPSS/PC+ program, with the following tests: the Kolmogorov-Smirnov test to determine how well the data fit a normal distribution; and the t-test to test the difference between the means of two independent or paired samples (t-test groups and t-test pairs). Differences between the three groups were tested by the Kruskal-Wallis one-way analysis of variance. The degree of association between lymphocyte proliferation and IFNγ production was calculated by the coefficient of correlation.

RESULTS

Figure 1 shows the lymphocyte proliferation index in tuberculous and nontuberculous patients. In tuberculous pleuritis, the pleural lymphocyte response (9.4 ± 4.3) was significantly higher than that of peripheral lymphocytes (5.1 ± 2.2), whereas in tuberculin-positive nontuberculous patients, the peripheral lymphocyte response (6.2 ± 2.3) was higher than that of pleural lymphocytes (3.9 ± 1.7). Tuberculous pleural fluid lymphocytes underwent greater blastic transformation than pleural lymphocytes of tuberculin-positive nontuberculous patients, whereas the opposite occurred in peripheral lymphocytes, with a higher response in tuberculin-positive nontuberculous patients, although in this case the difference was not significant. In tuberculin-negative nontuberculous patients, there was no lymphoblastic response in either the pleural fluid or peripheral blood.

The IFNγ stimulation index in the different groups is shown in Figure 2. The IFNγ production and lymphocyte transformation index were concordant. Thus, in tuberculous pleuritis, pleural lymphocyte IFNγ production (374 ± 549) was higher than that of peripheral lymphocytes (71 ± 106), whereas in tuberculin-positive nontuberculous patients the opposite occurs, with production being greater in peripheral lymphocytes (92 ± 103 vs 36 ± 37). Similarly, tuberculous pleural lymphocytes produce more IFNγ than tuberculin-positive nontuberculous lymphocytes, while no significant differences were found in peripheral blood.

In the two patients with tuberculous pleuritis who had a negative tuberculin skin reaction, the lymphocyte transformation index of peripheral cells was 3 or less, and the IFNγ production index was 6 or less. In pleural fluid the lymphocyte transformation index was greater than 3 and that of IFNγ was greater than 6 in

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**Figure 2.** Index of gamma interferon production after PPD stimulation of pleural fluid and blood lymphocytes in patients with tuberculous pleural effusion (I), nontuberculous tuberculin-positive pleurisy (II), and nontuberculous tuberculin-negative pleurisy (III). In group 1, open circles indicate tuberculin-negative patients.
Table 1—Correlation between Lymphocyte Proliferation Index and Gamma-Interferon Stimulation Index in Different Groups

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<th>Group and Specimen</th>
<th>r*</th>
<th>r†</th>
<th>p Value</th>
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<td>Overall Pleural fluid</td>
<td>0.6519</td>
<td>0.4950</td>
<td>&lt;0.001</td>
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<td>Blood</td>
<td>0.5477</td>
<td>0.3001</td>
<td>&lt;0.001</td>
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<td>Tuberculous pleural</td>
<td>0.5715</td>
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<td>0.013</td>
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<td>Blood</td>
<td>0.2813</td>
<td>0.0791</td>
<td>0.274</td>
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<tr>
<td>Nontuberculous tuberculin-positive pleural</td>
<td>0.5632</td>
<td>0.3172</td>
<td>0.071</td>
</tr>
<tr>
<td>Blood</td>
<td>0.6342</td>
<td>0.4022</td>
<td>0.027</td>
</tr>
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* r, Coefficient of correlation.
† r, Coefficient of determination.

the two cases.

In both the pleural fluid and peripheral blood, there was little correlation between lymphocyte transformation and IFNγ production, although it was significant in some groups (Table 1).

**DISCUSSION**

T-Lymphocytes previously sensitized by an antigen undergo blastic transformation when stimulated by the same antigen and produce different lymphokines, including IFNγ. The production of IFNγ in response to viral antigens has been demonstrated in cytomegalo virus, herpes simplex virus, Epstein-Barr virus, vaccinia virus, mumps virus, and influenza virus infections.

Tuberculosis is one of those diseases in which delayed hypersensitivity response plays an important role. Pleural infection by Mycobacterium tuberculosis is characterized by a lymphocytic infiltrate, with the formation of a T-lymphocyte-rich exudate. In vitro stimulation of these lymphocytes with PPD leads to their proliferation and IFNγ production. In tuberculous pleuritis, our results agree with those in the literature and confirm that the pleural lymphocyte response is much greater than that of peripheral lymphocytes in both blastic response and IFNγ production.

In two patients with pleural tuberculosis, anergy was associated with lack of response of blood lymphocytes to in vitro stimulation with PPD, whereas peripheral lymphocyte blastic response and IFNγ production were elevated. This phenomenon has been attributed either to preferential sequestration of antigen-specific T-lymphocytes into the pleural space or to the presence of suppressor cells in blood. In our country a high percentage of the population has a positive tuberculin reaction, with no active tuberculosis. Nontuberculous patients with a positive tuberculin test have lymphocytes previously sensitized to tuberculous antigens which should react to “in vitro” PPD stimulation; however, in no publication has lymphocytic response in tuberculin-positive nontuberculous patients been studied, except for two patients with carcinosis pleurae, who exhibit a lower blastogenic response in the pleural fluid in contrast to the patients with tuberculous pleurisy. In our previous studies, we found no significant differences in pleural fluid characteristics of tuberculous and nontuberculous patients with respect to the proportion of CD3+, CD4+, and CD8+ lymphocytes or to the proliferative response of these lymphocytes to stimulation with phytohemagglutinin, concanavalin A, and pokeweed mitogen. On the contrary, we did find very important differences in IFNγ levels in pleural fluid, which were high in all tuberculous pleural effusions and low in all nontuberculous pleural effusions, which would indicate specific IFNγ production by sensitized pleural lymphocytes stimulated in vitro by tuberculous antigens.

Herein we show that in tuberculin-positive patients with nontuberculous pleural effusion, “in vitro” lymphocytic response to PPD differs greatly from that of tuberculous pleuritis. Both the blastic response and IFNγ production are higher in peripheral than in pleural lymphocytes, contrary to what occurs in tuberculosis. Moreover, the tuberculous pleural lymphocyte response is higher than that of tuberculin-positive nontuberculous pleural lymphocytes.

These results concur with the concept of immunologic compartmentalization. In tuberculous pleuritis, there would be clonal expansion of PPD-responding T-lymphocytes in the pleural compartment, which would account for the greater response of pleural lymphocytes. This expansion of PPD-specific lymphocytes would not occur in nontuberculous pleuritis, but lymphocytes sensitized to other antigens would accumulate in the pleural compartment, which would explain why the response of these lymphocytes to PPD is lower than that of peripheral blood and tuberculous pleural lymphocytes.

The response of blood lymphocytes to PPD in tuberculous patients is lower than that in healthy tuberculous positive controls, which has been attributed to the presence of suppressor cells. We also observed a slightly higher response of peripheral lymphocytes in tuberculin-positive nontuberculous patients than in tuberculous patients, although the difference was not significant.

In all groups, we found concordance between the blastogenesis and IFNγ production; however, correlation between the two tests is very low (R²<0.50 in all cases). This concurs with the fact that both biologic processes point to cellular response “in vitro” but may be independent processes mediated by different cell subpopulations.
The lack of kinetic data about IFNγ production in our results is due to the established lymphoproliferative response to PPD in our laboratory. Almost all researchers use the same days of culture for IFNγ production and lymphoproliferative response, usually five-day or six-day culture.21,27,29

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