Purified Protein Derivative–Activated Type 1 Cytokine–Producing CD4+ T Lymphocytes in the Lung: A Characteristic Feature of Active Pulmonary and Nonpulmonary Tuberculosis

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Because tuberculosis (TB) is primarily a pulmonary disease, we examined the cytokine responses of CD4+ T lymphocytes in bronchoalveolar lavage (BAL) fluid after incubation with purified protein derivative (PPD) in human immunodeficiency virus–negative patients with TB and control subjects with nontuberculous respiratory disease. Parallel blood and BAL fluid samples from each subject were incubated with or without PPD, and the proportions of CD4+ T lymphocytes producing interferon (IFN)–γ or tumor necrosis factor (TNF)–α were measured by flow cytometry. The proportions of PPD-activated IFN-γ– and TNF-α–producing CD4+ cells were low among control subjects (median, 0.33% and 0.78%, respectively). By contrast, among patients with TB, strong IFN-γ and TNF-α responses were demonstrated (median, 24.0% and 32.4%, respectively), regardless of whether the TB was pulmonary or nonpulmonary. Measurement of type 1 cytokine production by CD4+ T lymphocytes in response to PPD in BAL fluid is a promising new diagnostic test for active TB in immunocompetent individuals.

It is estimated that one-third of the world’s population is infected with Mycobacterium tuberculosis and that there are 8 million new cases of tuberculosis (TB) and nearly 3 million TB-related deaths each year [1]. The most common route of infection is through the inhalation of droplets carrying the mycobacterium. This results in a local lung immune response that generally contains the infection. However, reinfection or reactivation may occur, which results predominantly in apical lung disease [2].

The reference-standard diagnostic test for TB remains the visualization of acid-alcohol–fast bacilli by Ziehl-Neelsen or auramine staining, with confirmatory culture of the organism. This can take up to 8 weeks when solid culture medium is used, although Bactec systems (Becton Dickinson) may achieve more-rapid culture results [3, 4]. However, only 54% of all cases of TB and 61% of cases of pulmonary TB were culture positive in 1999 in the United Kingdom [5]. Although the true figures for culture-positive TB may be higher than these (because of underreporting), there still remains a large proportion of TB diagnoses that are made on clinical grounds alone. More recently, DNA amplification techniques have been used as a rapid diagnostic test in cases of suspected TB [6–8]. However, the sensitivity of DNA amplification tests may be reduced in individuals with smear-negative disease [9, 10].

An alternative diagnostic strategy is suggested by the discovery that antigen-specific cells can be identified by a variety of different techniques [11–14]. Nevertheless, these studies have thus far been almost exclusively di-
rected toward examining responses in the blood. We reasoned that, in infectious lung diseases, antigen-specific responses of lymphocytes recovered from bronchoalveolar lavage (BAL) fluid might prove to be clinically more relevant. We used flow cytometry (FCM) to detect CD4+ lymphocyte cytokine production in response to purified protein derivative (PPD) in short-term cultures of blood and BAL fluid from patients with suspected TB.

PATIENTS, MATERIALS, AND METHODS

Patients. Of the 36 patients included in our study, 22 received diagnoses of TB; 15 had pulmonary disease, 4 had disseminated disease, and 3 had nonpulmonary disease (table 1). The diagnoses for all of the patients with TB was confirmed by culture, including that from aspirates of bone (patient 1) and lymph nodes (patients 2 and 3). Patients with disseminated TB predominately had cerebral (patient 4) and lymph node (patients 6 and 7) disease. Patient 5 had miliary shadowing of the chest radiograph and culture-confirmed TB meningitis. Results of testing of blood samples, but not of BAL fluid, were available for 5 other patients who had culture-positive pulmonary TB. All patients with TB had negative results of testing for human immunodeficiency virus (HIV).

The control group was composed of 14 patients with a variety of conditions requiring diagnostic bronchoscopy for which TB was considered in the differential diagnosis (table 1). Of the 2 patients with cytomegalovirus infection, one had chronic renal failure, and the other was a bone marrow transplant recipient. For 2 patients, no diagnosis was determined through bronchoscopy, but the symptoms resolved. Three control subjects (patients 29, 30, and 31; table 1) had negative results of testing for HIV, and the remainder were not tested. The untested patients were generally older and were not considered to be in a high–HIV risk group. Moreover, all absolute blood CD4+ cell counts measured for these patients as part of the study were >500 cells/μL. Five of these patients had been vaccinated with bacille Calmette-Guérin (BCG); 9 had not. In addition, blood was obtained from 20 BCG-vaccinated healthy control subjects who were nursing, medical, and laboratory staff at the Royal Free Hospital (London); 11 members of this group were men, and the median age was 36 years (range, 22–53 years). This group did not undergo BAL.

BAL. BAL was done by a standard technique, using a flexible bronchoscope wedged into a subsegmental bronchus while the patient was under intravenous sedation. Aliquots of warmed sterile normal saline were introduced through the bronchoscope and aspirated into a siliconized glass bottle. An area of lung that showed signs of TB on radiologic examination was washed; if no signs of TB were seen on radiologic examination, the right middle lobe was used. For 4 patients who had received a previous microbiological diagnosis of TB, bronchoscopy was performed within 2 weeks after the commencement of TB therapy. In the remaining cases, TB therapy was initiated after bronchoscopy.

Sample preparation. The fresh BAL fluid sample was placed on ice, and samples were sent to the relevant diagnostic laboratories. The remaining BAL fluid (25–50 mL) was centrifuged at 430 g for 8 min, decanted, filtered through a 100-μm filter (CellTricks; Partec), and then centrifuged again. The pellet was resuspended in 1 mL of culture medium (RPMI 1640 medium containing 10% fetal calf serum). Aliquots of BAL fluid in culture medium were then analyzed by FCM to assess the lymphocyte percentage, the ratio of CD4+ to CD8+ cells, and the absolute CD4+ cell count before the sample was cultured with PPD.

FCM analysis of lymphocytes in BAL fluid and blood. CD45–fluorescein isothiocyanate (FITC; Becton Dickinson) was added to 25 μL of the BAL fluid suspension for staining at room temperature in the dark for 15 min. A second 25-μL BAL fluid aliquot was stained with a cocktail of the following monoclonal antibodies: CD4-FITC (Royal Free Hospital), CD8-phycocerythrin (PE; Royal Free Hospital), and CD3-PE/CD5 (Dako). After staining, PBS was added to each tube to a volume of 1 mL. In addition, 50 μL of whole blood collected from each patient in lithium heparin tubes was stained as described above, with the substitution of lysis buffer (NH4Cl) for PBS. All antibodies were used in pretitrated optimal concentrations. Fresh BAL fluid and blood samples stained with CD45 and CD4/CD8/CD3 were run on a Cytoron Absolute flow cytometer (Ortho Diagnostics). A CD45+ panleukogate was used to count the absolute number of all leukocytes [15], and lymphocytes were identified by their strong CD45 staining and lymphoid scatter characteristics (figure 1). The number of CD4+ cells and the ratio of CD4+ to CD8+ lymphocytes in BAL fluid and blood were calculated for the second tube.

PPD stimulation and FCM analysis. Aliquots of the BAL fluid suspension containing 1 × 105 CD4+ lymphocytes in 1 mL of culture medium were placed into 2 sterile 5-mL polypropylene tubes (Thermo Life Sciences). One milliliter of peripheral blood that had been collected in lithium heparin tubes from the same patient was placed into each of 2 polypropylene tubes. Ten micrograms of PPD (Statens Serum Institute) was added to 1 BAL fluid sample and 1 blood sample. The other tubes were unstimulated control samples. For 5 patients, 0.08 IU of tetanus toxoid (Pasteur Merieux) was added to a third BAL fluid sample as a control antigen. The samples were incubated for 2 h at 37°C and 5% CO2, and then 5 μg of brefeldin A (Epitope Technologies) was added, and the samples were incubated for 14 h more.

After incubation, the samples were vortexed vigorously to detach cells from the walls of the tube. First, lymphocyte surface markers were stained using CD4-FITC and CD3–peridinin chlo-
Table 1. Demographic characteristics and results of diagnostic testing for patients with tuberculosis (TB) and nontuberculous respiratory conditions.

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<td>No diagnosis</td>
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NOTE. A, Asian; AFB, acid-fast bacilli; BA, black African; BAL, bronchoalveolar lavage; BCG, bacille Calmette Gue´rin; BUK, black, born in the United Kingdom; IFN, interferon; ND, not done; O, other; PCR, polymerase chain reaction; PPD, purified protein derivative; W, white.

a Diagnosis of TB from the BAL fluid specimen by AFB smear, PCR, and culture. Patients 1, 2, and 3, who had nonpulmonary TB, were diagnosed through testing of tissue biopsy specimens, all of which were smear and PCR negative but culture positive.

b For some patients with TB, antituberculous therapy was initiated before a diagnosis was made through BAL fluid and PPD testing. The no. of days after the start of TB therapy was 8 for patient 1, 3 for patient 2, 14 for patient 4, 8 for patient 10, and 7 for patient 16. For all other patients with TB, antituberculous therapy was started after BAL.

c Percentage of lymphocytes in the total BAL fluid leukocyte population.

d Percentage of CD4+ lymphocytes producing IFN-γ after incubation with PPD in BAL fluid.

e Percentage of CD4+ lymphocytes producing IFN-γ after incubation with PPD in blood.

Respiratory conditions.

Ifn, interferon; ND, not done; O, other; PCR, polymerase chain reaction; PPD, purified protein derivative; W, white.
Figure 1. Flow cytometric dot plots for a patient with tuberculosis who had a low lymphocyte percentage (A) and lymphocytosis (B) in bronchoalveolar lavage fluid. The vertical axis of each dot plot distinguishes CD45<sup>+</sup> leukocytes from nonleukocyte debris. The CD45<sup>+</sup>-panleukogate is denoted “R1.” Lymphocytes form a homogenous population with low side scatter (SSC), denoted “R2.” Lymphocyte percentages are calculated as the proportion of R2 events within R1.

(Becton Dickinson); 40,000 CD4<sup>+</sup> events were acquired, and the proportion of IFN-γ- and TNF-α–staining cells within the total lymphocyte and CD4<sup>+</sup> T cell populations were analyzed in both the PPD-activated and the control cultures, using WinMDI software (version 2.8; J. Trotter [http://facs.scripps.edu/software.html]). The responses attributable to specific PPD effects were calculated by subtracting the responses in the control tubes from those in the PPD-activated tubes (figure 2).

**Statistical analysis.** Median values and absolute ranges were recorded, and nonparametric statistical analysis was performed using the Mann-Whitney U test to determine the statistical difference between data sets.

**RESULTS**

**Lymphocyte percentages and ratios of CD4<sup>+</sup> to CD8<sup>+</sup> cells in BAL fluid.** BAL fluid samples from most of the patients with TB showed significant lymphocytosis (median, 28.3%; range, 0.7%–70.4%; table 1). In 3 patients (patients 19, 20, and 21), low lymphocyte percentages were present in BAL fluid. In 2 of these individuals (patients 20 and 21), washings were taken from apical cavities where pus was aspirated; therefore, the vast majority of cells were neutrophils.

It was interesting that lymphocytosis was present in BAL fluid from patients with pulmonary TB (median, 20.6%) and patients with nonpulmonary TB (median, 32.0%). The ratios of CD4<sup>+</sup> to CD8<sup>+</sup> cells in BAL fluid (table 1) in the TB group were highly variable (range, 0.4–11.7), although the median ratio (2.6) was within the range for healthy control subjects [17].

When patients without TB were investigated, the lymphocyte percentages and ratios of CD4<sup>+</sup> to CD8<sup>+</sup> cells in BAL fluid were also highly variable, as would be expected in such a heterogenous group. The median lymphocyte percentage in this group was 21.2% (range, 2.0%–81.7%). The highest lymphocyte numbers were seen in 3 patients with sarcoidosis (median, 76.0%), which is in agreement with the documented abnormalities associated with that disease. The ratio of CD4<sup>+</sup> to CD8<sup>+</sup> cells in the control group was also variable (median, 1.7; range, 0.5–43.4).

**Comparison of IFN-γ and TNF-α responses to PPD in BAL fluid from patients with TB and control subjects without TB.** The median percentage of CD4<sup>+</sup> lymphocytes producing IFN-γ in response to PPD in BAL fluid samples from patients with TB was high (24.0%; range, 1.98%–37.2%). Even stronger TNF-α responses were noted in samples from these patients (median, 32.4%; range, 3.81%–47.4%). FCM analysis determined that both cytokines were produced mainly by the same activated CD4<sup>+</sup> lymphocyte population. Strong IFN-γ responses to PPD by CD4<sup>+</sup> cells in BAL fluid were demonstrated in patients with TB who had pulmonary disease (median, 21.8%) and in those who had nonpulmonary/disseminated disease (median, 24.4%). TNF-α responses in BAL fluid for these 2 groups were also similar.

By contrast, the percentage of IFN-γ–producing CD4<sup>+</sup> cells in BAL fluid from patients with nontuberculous respiratory disease was low (median, 0.33%; range, 0.01%–1.12%; figure 3). These results were highly statistically significant (P < .0001), compared with those for patients with TB. TNF-α responses to PPD by CD4<sup>+</sup> cells in BAL fluid from control subjects were also weak (median, 0.78%). Values for control subjects were low, regardless of whether the patients had been vaccinated with BCG.

**Further characterization of the cytokine responses to PPD in BAL fluid from patients with TB.** During the short incubation period of our assay, PPD was demonstrated to activate mainly CD4<sup>+</sup> lymphocytes, and not CD8<sup>+</sup> T lymphocytes (figure 2). The PPD-activated CD4<sup>+</sup> cell populations in the lungs
TB Antigen–Specific Lymphocytes in the Lung

Figure 2. Flow cytometric dot plots for a patient with tuberculosis, demonstrating cytokine synthesis in response to purified protein derivative (PPD) after 16 h of incubation. A, Proportion of CD3+ lymphocytes producing interferon (IFN–γ) in bronchoalveolar lavage (BAL) fluid after incubation with PPD. B, Tumor necrosis factor (TNF–α) response to PPD in BAL fluid. C, IFN–γ response in BAL fluid when no antigen (Ag) is added. D, Proportion of CD3+ lymphocytes producing IFN–γ in blood after incubation with PPD. The vertical axis in each dot plot describes a population of CD4+ T lymphocytes (upper left quadrants) and CD3+CD4+CD8– T lymphocytes (lower left quadrants). The upper right quadrants show the cytokine response of CD4+ T cells, and the lower right quadrants demonstrate the cytokine response of CD8+ T lymphocytes. A and B show strong cytokine responses by CD4+ cells and limited responses by CD8+ cells. The responses in the BAL fluid control (C) and the blood sample (D) were weak.

remained large in patients who had received antituberculous therapy for up to 2 weeks (median, 8 days) before BAL (patients 1, 2, 4, 10, and 16; table 1). In addition, steroid therapy did not significantly attenuate the response in patient 4, who was treated with corticosteroids for 10 days before BAL.

To investigate whether the induction of cytokine responses was really antigen specific, tetanus toxoid was added as a control antigen to BAL fluid samples from 5 patients with TB. The responses were very weak and similar to those in the control tubes to which no antigen was added, thus confirming that the reactions were PPD-antigen specific.

Our results demonstrate that strong cytokine responses to PPD are present throughout the lung and are not localized to areas of lung tissue in which radiologic abnormalities are seen in patients with TB. In patients with nonpulmonary TB, BAL was performed from the right middle lobe, which radiologic examination showed to be unaffected, and in each case, strong responses to PPD were noted (table 1). In addition, washings from areas of affected and unaffected lung were performed for selected patients with pulmonary TB, and comparative analysis showed strong responses in washings from both areas (data not shown).

Comparison of IFN–γ and TNF–α responses in blood samples from patients with TB and from BCG-vaccinated control subjects. We next investigated whether there were differences
between the PPD responses of CD4+ cells in blood samples from patients with TB and the responses of cells in samples from a group of healthy BCG-vaccinated control subjects (figure 4). The median percentage of CD4+ T lymphocytes producing IFN-γ in response to PPD among patients with TB was 0.07% (range, 0.01%–1.43%). This value was lower than that for the BCG-vaccinated control subjects (median, 0.14%; range, 0.01%–0.91%), and there was no statistically significant difference between the values for the 2 groups (P = .80). Similarly, the CD4+ TNF-α responses in blood samples from patients with TB were weak (median, 0.18%), as were those in samples from BCG-vaccinated control subjects (median, 0.32%).

DISCUSSION

The demonstration of strong IFN-γ and TNF-α synthesis responses to PPD by CD4+ cells in BAL fluid, compared with the weak responses in peripheral blood, is powerful evidence for the dominance of lung immune responses and for the active recruitment of TB-specific CD4+ T lymphocytes to the lung during TB infection. Other human studies [18–20] and murine studies [21, 22] also have indicated that lung immune responses predominate during TB infection in different experimental systems. In 2 of the human studies [18, 19], T cells were separated from the leukocytes in BAL fluid and incubated with peripheral blood mononuclear cells and TB antigens, and then [3H]thymidine incorporation was measured. Those studies used complex experimental techniques because of concerns about the possible suppressive effects of alveolar macrophages and their unsuitability as antigen-presenting cells (APCs). The impressive antigen-specific CD4+ lymphocyte cytokine responses demonstrated in our simplified system supports the findings of other investigators that BAL fluid contains effective APCs [23, 24].

The main conclusion of our study is that the PPD-activated CD4+ T cell type 1 cytokine response, measured by IFN-γ or TNF-α synthesis, appears to be a useful measure for diagnosis of active TB in HIV-uninfected individuals. It is of considerable interest that strong type 1 cytokine responses by CD4+ cells were present not only in patients with pulmonary disease but also in 3 patients with nonpulmonary TB and 4 patients with disseminated TB in whom extrapulmonary complications dominated the clinical picture. This finding has particularly significant diagnostic implications, because obtaining BAL fluid may be both easier and safer than biopsy of other tissues in patients who are suspected of having occult TB.

The other significant development inherent in our assay is that the results were available within 24 h of acquisition of the BAL fluid sample. It is relevant that 55% of patients in our TB cohort were smear negative and 29% were both smear and PCR negative. Thus, the immunodiagnostic test avoided the delays associated with waiting for results of a culture for M. tuberculosis.

One patient with TB had a weak (1.98%) response to PPD by CD4+ cells in BAL fluid. This patient was asymptomatic and afebrile, with normal inflammatory markers, but was referred for further investigation because of abnormalities seen on a chest radiograph. The unusual findings of a normal lymphocyte percentage and a weak PPD response in BAL fluid...
suggest that this patient may have had a very early reactivation of TB that occurred during the recruitment of antigen-specific lymphocytes to the lung.

A corollary of our observations is that testing of the CD4⁺ cell responses to PPD in blood alone is inadequate for diagnosis of active TB. The blood lymphocyte responses in patients with TB remained indistinguishable from the responses seen in BCG-vaccinated control subjects (figure 4). The most likely explanation for the weak responses seen in blood samples from patients with TB is that antigen-specific lymphocytes are actively recruited to the site of infection in the lung, resulting in relative depletion in the blood. Furthermore, it has been demonstrated in an ELISPOT system that BCG-vaccinated subjects develop cytokine responses to PPD [25]. Therefore, PPD, which shares epitopes with both M. tuberculosis and BCG, is not a discriminating stimulatory antigen for measurement of blood lymphocyte responses. Interestingly, BCG vaccination did not provoke a significant cytokine response to PPD in BAL fluid—no difference was demonstrated between the responses in BCG-vaccinated and -unvaccinated control subjects (table 1).

One could argue that TB infection and BCG vaccination can be better discriminated by using antigens specific for TB, such as the TB early secretory antigen ESAT-6. Overlapping peptides of this antigen have been demonstrated to elicit IFN-γ responses in peripheral blood mononuclear cells from patients with TB but not from BCG-vaccinated subjects when a sensitive ELISPOT technique was used for measurement [13]. However, this test did not distinguish between active and latent disease. Furthermore, the use of ESAT-6 does not solve the problem of impairment of cytokine responses in blood from patients with severe TB [26, 27]. These pitfalls are avoided by examining lung immune responses, in which strong cytokine responses by TB antigen–recruited cells, in addition to a generalized lymphopenia, support active TB.

The FCM assay used in our study has a considerable advantage over other methods of determining antigen-specific responses, such as the ELISPOT system, for 3 reasons. First, FCM provides a rapid and precise quantification of the total lymphocyte percentage in BAL fluid, together with the proportions and absolute counts of the responding cell types (CD4⁺ and CD8⁺). Second, different cytokine responses—in our study, those of IFN-γ and TNF-α—can be investigated in the same activated lymphocytes. Third, the new generation of cheap red diode laser flow cytometers will render FCM technology an accessible and affordable option [28]. Nevertheless, fiberoptic bronchoscopy is an expensive diagnostic tool, and this sort of study will need to be performed using cheap nonbronchoscopic methods of obtaining a lavage sample [29], if these techniques are to be useful in resource-poor contexts.

From an immunologic perspective, the present study highlights several issues. The importance of the type 1 cytokine axis in the control of TB has been documented both in murine experiments [30–32] and in patients with inherited defects in the interleukin-12 and IFN-γ receptors [33–35]. Other investigators, who used murine models of TB, have demonstrated that TNF-α is essential for the generation of protective granulomas, without which TB control is insufficient [36, 37]. Our study gives direct support for the idea that both IFN-γ and TNF-α mediate antituberculous responses in the lung. It is intriguing that powerful cytokine responses to PPD in BAL fluid are generated in patients with nonpulmonary TB. These findings suggest that a lymphocyte recirculation pathway to the lung exists, which presumably reflects the fact that, even in these patients, the origin of postprimary disease was the lung.

Finally, the data presented here demonstrate the limitations of exclusive measurement of CD4⁺ lymphocyte responses. In patients coinfected with HIV and M. tuberculosis, CD4⁺ lymphopenia may limit the applicability of our test for IFN-γ responses by CD4⁺ cells. To develop a reliable immunodiagnostic TB test for this important group of patients, antigen-specific CD8⁺ cell responses to TB antigens will need to be explored in the lung. Furthermore, the specificity of this method for identifying active TB will also need to be explored by examination of the responses to PPD in BAL fluid from patients with treated disease. In our study, 1 patient (patient 8; table 1) underwent a second bronchoscopy immediately after the completion of TB therapy. The proportion of PPD-activated IFN-γ-producing CD4⁺ lymphocytes after therapy was 0.45%, compared with 12.4% at the time of TB diagnosis. Whether antigen-specific responses in BAL fluid always decrease to low levels after therapy will need to be confirmed in a larger number of patients. In addition, the applicability of this test for determining which patients have latent and which have active TB will also need to be explored. Finally, a more extensive investigation of responses to PPD in BAL fluid from patients with nontuberculous lung disease will be needed before firm conclusions about the specificity of this test can be drawn.

In conclusion, we have described a novel method for diagnosing TB by measuring intracellular cytokine responses to PPD in BAL fluid by a simple and rapid FCM technique. We have demonstrated strong responses in BAL fluid from patients with both pulmonary and nonpulmonary TB but weak responses in BAL fluid from patients with nontuberculous respiratory disease. Therefore, the test appears to be a promising diagnostic resource, particularly for patients with nonpulmonary TB, for whom achieving a culture-based diagnosis may be both difficult and hazardous.

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