Relation of bronchoalveolar lavage T lymphocyte subpopulations to rate of regression of active pulmonary tuberculosis

C-T Yu, C-H Wang, T-J Huang, H-C Lin, H-P Kuo

Abstract

Background — Effective host defence against mycobacterial infection chiefly depends on the interactions between macrophages and T lymphocytes. This study investigated the relation of cellular components and their activity of cells obtained by bronchoalveolar lavage (BAL) from the lower respiratory tract to disease regression in patients with active pulmonary tuberculosis without HIV infection.

Methods — Clinical indices including age, sex, the presence of diabetes, fever, the presence of resistant strains of mycobacteria, the bacterial load in sputum, and disease extent on chest radiography at presentation were assessed before commencing four-drug antituberculosis therapy. Twenty two patients with active pulmonary tuberculosis were divided into rapid, intermediate, and slow regression groups. Subpopulations of alveolar macrophages separated using discontinuous Percoll density gradient centrifugation and T lymphocytes (with CD3, CD4, CD8, and CD25 monoclonal antibodies) were quantified.

Results — There were no differences among rapid, intermediate, and slow regression groups in terms of age, sex, the presence of diabetes, the presence of resistant strains of mycobacteria, or the bacterial load in sputum. No differences were found between the groups in terms of subpopulations of alveolar macrophages or numbers of CD3 and CD4 lymphocytes. By contrast, an increase in CD8 cells was shown in the slow regression group compared with the rapid and intermediate regression groups. CD25 cell numbers were increased in the rapid regression group compared with the slow regression group. The CD4/CD8 ratio was decreased in the slow regression group compared with the rapid and intermediate regression groups and the relation between the proportion of CD25 cells and the CD4/CD8 ratio in BAL fluid was significant.

Conclusions — A decreased CD4/CD8 ratio with an increase in CD8 cells in the alveolar spaces was associated with slow disease regression in patients with active pulmonary tuberculosis without HIV infection, suggesting that the balance of T lymphocyte subsets may play a central part in the modulation of host defence against mycobacterial infection.

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Keywords: pulmonary tuberculosis, bronchoalveolar lavage, alveolar macrophage, T lymphocyte subpopulations.

Tuberculosis remains the leading cause of death in the world and accounts for 6–7% of all deaths in the developing world. Cell-mediated immunity plays a critical part in eliminating mycobacteria. It is believed that mycobacteria inhaled into the airways are phagocytosed by alveolar macrophages and then processed for antigen presentation to T lymphocytes; lymphokines released from activated lymphocytes may prime macrophages to inhibit mycobacterial replication within macrophages. Host defence against mycobacteria therefore chiefly depends on the interactions between macrophages and T lymphocytes for effective control of infection. More than 95% of individuals affected by pulmonary tuberculosis can be cured with various antituberculous drugs introduced in recent years. However, the pattern of resolution of pulmonary tuberculosis differs between patients, suggesting that individual variations in cellular immunity against mycobacterial invasion may be an important factor in determining the rate of mycobacterial eradication. This is especially the case in patients with pulmonary tuberculosis and HIV infection.

Short six month courses of antituberculous chemotherapy are now widely used throughout the world, and it is possible that certain individuals might require more intensive treatment. However, there are no data to indicate for whom this more protracted treatment should be considered. Since host defence against mycobacterial invasion occurs within bronchoalveolar spaces, bronchoalveolar lavage (BAL) could provide a valuable insight into the magnitude of the individual cellular immune response which may be an important determinant of the prognosis and this may lead to a better approach to treatment of active pulmonary tuberculosis.

Recent reports have investigated peripheral blood and pleural fluid, but less is known of BAL cells. We have therefore evaluated the relationship of BAL cells recovered from the lower respiratory tract and their activity status with clinical indices in patients with active
pulmonary tuberculosis without HIV infection before or within one week of antituberculous treatment.

Methods

STUDY POPULATION
Twenty two patients with active pulmonary tuberculosis (15 men and seven women, including 13 diabetics) with a mean (SE) age of 47.7 (3.6) years were enrolled in this study. All were negative for HIV infection. The nutritional status of each patient was assessed, including measurement of body mass, height, triceps skinfold thickness, mid arm circumference, and serum albumin level. Patients in poor nutritional status (body mass <90th percentile or mid arm circumference and triceps skin fold thickness <25th percentile) were excluded. None of the patients was taking corticosteroids or other immunosuppressant medication.

CLINICAL INDICES
Clinical indices including age, sex, the presence of diabetes, fever, the presence of resistant strains of mycobacteria, the bacterial load in the sputum, and disease extent on chest radiography at presentation were assessed. Fever was defined as an initial presentation with a temperature of >37.5°C, persisting for more than five days, which subsided on starting antituberculous therapy, together with the exclusion of common bacterial or viral infection.

SPUTUM ASSESSMENT
The patients were encouraged to cough deeply to expectorate a specimen of lower respiratory tract secretions suitable for microscopic examination and culture. If contaminated saliva or large oropharyngeal squamous cells were predominant, the aliquot was discarded. The bronchopulmonary origin of the specimen was assured if it contained mucous strands in which inflammatory polymorphonuclear cells, monocytes, macrophages, and bronchial epithelial cells were embedded. In all patients early morning sputum collected freshly on three different days was centrifuged and the sediment was stained for acid fast bacilli by the auramine fluorochrome procedure after an examination to assure its bronchopulmonary origin. Bacterial numbers were determined by counting 30 fields under oil immersion and were graded by an independent experienced technician into four categories: (0) absence of bacilli; (1) 1–9 bacilli; (2) 10–29 bacilli; and (3) >30 bacilli per 30 oil-immersed fields. The bacterial gradings from specimens collected on three days were totalled and presented as an index of sputum bacterial load. In all patients at least one sputum specimen was positive for acid fast bacilli on microscopic examination and grew Mycobacterium tuberculosis. Sputum conversion was assessed in the first 3–6 months and, occasionally, up to 9–12 months if a sputum sample was available. The presence of resistant strains was recorded according to the final culture results. Multidrug-resistant strains are defined here as M tuberculosis resistant to isoniazid and rifampicin, with or without resistance to other drugs, as previously described.11

GRADING OF DISEASE EXTENT ON CHEST RADIOGRAPHY
Posteranterior chest radiographs were taken of all patients at the time of hospital admission and every three months thereafter for at least nine months. A grading of the extent of disease proposed by the World Health Organisation (1960) was adopted to assess the severity of disease at presentation12: 0 = no involvement; 1 = trivial; 2 = slight; 3 = limited; 4 = moderate; 5 = extensive; and 6 = gross.

PATIENT GROUPING
The study population was allocated to one of three groups according to the resolution of pulmonary lesions: (1) rapid regression group (RR) showing more than 50% improvement in the extent of disease on chest radiography within three months of commencing treatment, and either complete resolution within nine months of treatment or residual fibrotic lesions which were unchanged for at least three months after cessation of treatment; (2) intermediate regression group (IR) showing 50% improvement in the extent of disease on chest radiography after 3–6 months of treatment, and either complete resolution within nine months of treatment or residual fibrotic lesions which were unchanged for at least three months after cessation of treatment; and (3) slow regression group (SR) showing less than 50% improvement in the extent of disease on chest radiography after six months of treatment, and either persistent active pulmonary lesions or incomplete resolution within nine months of treatment. To avoid observer bias the radiographs were initially assessed independently by two pulmonary physicians who were not aware of the laboratory results or clinical presentations, and they arrived at the same grading for 19 of 22 patients. Two patients were assessed IR by one observer and RR by the other; one patient was assessed RR by one observer and IR by the other. After discussion, consensus agreement was reached for the groupings for these three patients. Assessment throughout the study was also performed blind.

ANTITUBERCULOUS TREATMENT
All 22 patients received four-drug antituberculous chemotherapy including isoniazid (5 mg/kg/day), rifampicin (10 mg/kg/day) and ethambutol (20 mg/kg/day) for at least nine months, as well as pyrazinamide (25 mg/kg/day) for the initial two months of treatment. All patients took medication regularly without interruption.

PREPARATION OF BAL CELLS
BAL was performed before or within one week of antituberculous treatment using five aliquots
(50 ml each) of 0.9% saline solution as described previously. Briefly, sterile prewarmed (37°C) saline solution was introduced into the involved segment(s). The lavage fluid was then recovered by gentle aspiration and pooled and filtered through two layers of sterile gauze. The total cell count was evaluated on an aliquot of the pooled fluid using a haemocytometer. Differential cell counts were determined from cytocentrifuge preparations with modified Wright-Giemsa staining. The remaining fluid was centrifuged at 600 g for 20 minutes at 4°C. The cell pellet was then washed sequentially and resuspended in RPMI-1640 (Flow Laboratories, Paisley, UK) at 10⁶ cells/ml. Informed consent for bronchoscopy was obtained from all patients.

DENSITY FRACTIONATION OF ALVEOLAR MACROPHAGES

Alveolar macrophages were separated using discontinuous Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation as previously described. Isotonic Percoll was prepared by mixing stock Percoll with 9% saline solution at ratios calculated to produce working Percoll of specific gravities 1-030, 1-040, 1-050, and 1-070 g/ml. Specific gravities were checked with a refractometer before centrifugation. The alveolar macrophages (5 x 10⁶ in 5 ml RPMI-1640) were layered onto the top Percoll layer in a centrifuge tube containing 5 ml aliquots of the four Percoll solutions (least dense to most dense, top to bottom) and then centrifuged for 20 minutes at 400 g at 20°C. The cells that localised at each fractional interface were collected and named for the density upon which it floated. Total and differential cell counts were made from each layer. Proportional recovery of a given interface was defined as the number of cells recovered from the gradient expressed as a ratio of total cells recovered so that the sum of cells recovered totalled 1-0. All fractions comprised >95% alveolar macrophages and viability was determined by trypan blue dye exclusion. More than 90% of the cells recovered from the column after centrifugation were found in five fractions, with the remainder of the cells (lymphocytes, granulocytes, and eosinophils) being located in the pellet with specific gravity greater than the densest Percoll fraction used. Hypodense subpopulations were defined as cells with a specific gravity of <1-040 g/ml.

T LYMPHOCYTE SUBPOPULATIONS

T lymphocyte subpopulations were identified as CD3, CD4, CD8 or CD25 using monoclonal antibodies anti-Leu-4/CD3 FITC, anti-Leu-3a/CD4 FITC, anti-Leu-2a/CD8PE or anti-IL-2R-anti-Tac, respectively (Beckton Dickinson) and assayed by fluorescence-activated flow cytometry with a fluorescence-activated cell sorter (FACStar) (Beckton Dickinson).

STATISTICAL ANALYSIS

Results are expressed as means (SE). Statistical analysis of results was performed by the two tailed Student's t test for unpaired data. For data not normally distributed the Mann-Whitney U test was used. One-way analysis of variance followed by Bonferroni corrected t test or Kruskal-Wallis statistical analysis was performed when multiple comparisons were made. The relation between clinical indices, such as the disease extent on chest radiography or bacterial load in sputum, and T lymphocyte subpopulations in BAL fluid was sought by multiple regression analysis, then Spearman’s rank correlation test or linear regression analysis was applied to examine the significance of correlations between groups. χ² analysis was used for nominal data comparisons between two or three groups. A p value of <0.05 was considered significant.

Results

CLINICAL INDICES

Table 1 shows the clinical features of patients in the RR, IR, and SR groups. There were no significant differences among these groups in terms of age, sex, the presence of diabetes, the presence of resistant strains of M tuberculosis, or the bacterial load in the sputum. Patients in the RR group (62.5%) had fever at initial presentation compared with none of the five in the SR group (p<0.05). Although more patients in the IR group (n=4) were febrile than in the SR group, this did not reach statistical significance. The disease extent on chest radiography at initial presentation was significantly lower in the SR group (mean (SE) 3-6 (0.3)) than in the RR group (5.0 (0.3), p<0.02), but not significantly different from the IR group (4.0 (0.4)).

ALVEOLAR MACROPHAGE AND T LYMPHOCYTE SUBPOPULATIONS

Table 2 shows total cell numbers and subpopulations recovered by BAL in the three study groups. Total cell numbers and percentages of macrophages and lymphocytes were not significantly different among the groups, and there were no differences in proportions of alveolar macrophage subpopulations separated

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Table 1 Demographic data at presentation in three groups of patients with active pulmonary tuberculosis

<table>
<thead>
<tr>
<th></th>
<th>Rapid regression (n = 8)</th>
<th>Intermediate regression (n = 9)</th>
<th>Slow regression (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SE) age (years)</td>
<td>43.6 (6.5)</td>
<td>52.0 (5.6)</td>
<td>46.4 (7.4)</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>6:2</td>
<td>5:4</td>
<td>4:1</td>
</tr>
<tr>
<td>Diabetics</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Presence of drug-resistant tuberculosis:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no resistant strain</td>
<td>5</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>resistant to ethambutol</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>resistant to isoniazid</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>resistant to isoniazid and streptomycin</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>resistant to isoniazid and rifampicin</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Fever (% of patients)</td>
<td>5 (62.5%)</td>
<td>4 (44.4%)</td>
<td>* (0%)</td>
</tr>
<tr>
<td>Sputum bacterial load</td>
<td>3.7 (9.0)</td>
<td>3.2 (7.9)</td>
<td>2.6 (8.8)</td>
</tr>
<tr>
<td>Proportion score on chest radiograph</td>
<td>5.0 (0.3)</td>
<td>4.0 (0.4)</td>
<td>3.6 (0.3)**</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.02 compared with rapid regression group.
by density gradient or total and percentage of CD3 and CD4 T cells.

The five patients in the SR group had significantly higher percentages and total numbers of CD8 cells (40-4% (5-4%) and 1-5 (0-3) x 10^4 cells/ml, respectively) than the eight patients in the RR group (21-5% (3-2%) and 1-0 (0-1) x 10^4 cells/ml, respectively; p<0-01) and the nine patients in the IR group (23-8% (3-1%) and 1-0 (0-2) x 10^3 cells/ml, respectively; p<0-02). Patients in the RR group had a significantly higher proportion and total cell number of CD25 cells (10-8% (0-9%) and 0-5 (0-1) x 10^4 cells/ml, respectively) than those in the SR group (6-0% (0-9%) and 0-2 (0-1) x 10^3 cells/ml, respectively; p<0-01), but not those in IR group (8-9% (0-9%) and 0-4 (0-1) x 10^3 cells/ml). There was no difference in CD25 cell numbers between the IR and SR groups. The CD4/CD8 ratio was significantly lower in the SR group (1-4 (0-3)) than in the RR group (3-2 (0-4); p<0-02) and the IR group (2-5 (0-3); p<0-05), but there was no difference between the RR and IR groups.

**Radiographic Resolution and Sputum Conversion**

Most patients completely cleared their pulmonary lesions within nine months of four-drug antituberculous treatment except for three patients in the SR group. The time needed for complete resolution of pulmonary lesions on chest radiography was 6-4 (0-7) months in the RR group, 8-7 (0-6) months in the IR group, and 12-0 (1-3) months in the SR group.

The time needed for sputum conversion was significantly shorter in patients in the RR group (3-0 (0-9) months) than those in the IR group (4-3 (0-5) months; p<0-05) or the SR group (7-8 (0-7) months; p<0-01), and there was also a significant difference between the latter two groups (p<0-01).

**Relationships between Bronchoalveolar Profiles and Clinical Indices**

Three patients in the SR group failed to respond to antituberculous treatment. The pulmonary lesion was progressive in two of these three and stationary in the other, with persistent M. tuberculosis in the sputum after nine months of antituberculous treatment. In these three patients the CD4/CD8 ratio was strikingly lower (1-0 (0-1)) than in the other 19 patients (2-7 (0-3); p<0-02), and had a much higher proportion of CD8 cells (48-7% (2-7%)) than the other active tuberculous patients (23-3% (2-0%); p<0-001).

There was no correlation between the number of CD4, CD8 cells or CD4/CD8 ratio and the extent of disease on chest radiography, bacterial load in sputum, age, or the presence of diabetes. However, the proportion of CD25 cells was closely related to the CD4/CD8 ratio in BAL fluid (n=22, r=0-76, p<0-01) (figure).

**Discussion**

Our results have shown that patients with pulmonary tuberculosis with a lower CD4/CD8 ratio or higher number of CD8 cells in BAL fluid may have a delayed course of recovery. In the present study the resolution rate of pulmonary tuberculosis was determined by changes in the diffusion score on chest radiography, which may depend not only on mycobacterial activity but also on the tissue reaction. Resolution of disease on chest radiography therefore represents the overall response of the lungs to mycobacterial invasion. Patients with a more rapid recovery in the first 3-6 months of antituberculous treatment obtained sputum conversion and complete resolution of pulmonary lesions faster than the other groups. There were no differences in bacterial load or the presence of resistant strains of mycobacteria between patients with a rapid recovery and those with delayed resolution, suggesting that the host defence mechanism may be more important than bacterial virulence in eradicating mycobacterial invasion in patients without multidrug-resistant tuberculosis. Similarly, there was no difference in age, sex, or the presence of diabetes mellitus between patients showing a rapid recovery and those with de-
Regression of pulmonary tuberculosis and T lymphocyte subpopulations

layed resolution, indicating that these factors did not contribute to the recovery rate from pulmonary tuberculosis.

Host defence against mycobacteria depends chiefly on the interactions between macrophages and T lymphocytes. The macrophage is the effector cell in eradicating mycobacteria and is dependent on interactions with activated T lymphocytes.

The proportion of hypodense alveolar macrophages, an index of macrophage activation, is increased in patients with active pulmonary tuberculosis. In the present study we failed to identify any relation between total CD8 cell number or the subpopulations of alveolar macrophages defined by density and recovery rate from active pulmonary tuberculosis. The increase in the proportions of hypodense alveolar macrophages in our previous report and here may therefore represent a local response of macrophages to mycobacterial invasion without reflecting significant differences in prognosis.

T lymphocytes also play a central part in the cell-mediated immune defence against *M. tuberculosi*. Cytokines such as IL-2 or gamma interferon released from CD4 cells may activate macrophages to ingest and kill mycobacteria more effectively and to prime macrophages for massive release of tumour necrosis factor after ingestion of mycobacteria. Some of these cytokines secreted in tuberculosis are endogenous pyrogens that may induce fever. In the present study patients with a delayed or slow recovery were less febrile, which might suggest lower cytokine production that could reflect impaired interaction between T lymphocytes and macrophages. Suppressive lymphocytes have been identified in human tuberculous infections. The CD8 cell population may also represent functionally suppressive lymphocytes which downregulate the proliferation of CD4 cells and suppress the antymycobacterial action of macrophages. This could cause less tissue damage but at the expense of delaying clearance of the organisms.

An inverse relation between the recovery rate of miliary tuberculosis and CD8 cells in BAL fluid has been reported previously. Consistent with this, in our study the proportion as well as the total number of CD8 cells was significantly inversely related to the recovery rate with a predominance of CD8 cells seen in the SR group compared with the IR and RR groups. When CD8 cells comprised >40% of the total T lymphocyte number the time required for improvement in disease extent was more than six months and complete resolution exceeded nine months. It is possible that tuberculosis associated with a higher proportion of CD8 cells in BAL fluid results in a less efficient host defence response, possibly due to down-regulation of CD4 T cell and macrophage activity, leading to delayed resolution of disease.

The mechanism responsible for the increase in CD8 cells is unclear. An increase in CD8 cells with a reduced CD4/CD8 ratio has been reported in advanced or disseminated tuberculosis. A large bacterial load was reported to shift the T cell activation towards a predominance of CD8 cells. However, in the present study the disease extent (in terms of chest radiographic abnormality or bacterial load in sputum) was not related to numbers of CD4 or CD8 cells in BAL fluid.

Activated T lymphocytes may express IL-2 receptors on their cell membrane – that is, CD25 positive cells. A lower proportion of CD25 positive cells in patients with a lower CD4/CD8 ratio implies that T lymphocytes in those patients are less activated than in patients with a higher CD4/CD8 ratio. A significant increase in CD25 cells was found in patients with a rapid resolution of pulmonary tuberculosis compared with those with delayed resolution. The rate of recovery of active pulmonary tuberculosis therefore depends not only on the fine balance between CD4 and CD8 cells, but also the T lymphocyte activation status.

Recovery from tuberculosis, assessed by improvement in disease extent on chest radiography, therefore depends upon the host response to mycobacterial invasion, and this response may be more critical than bacterial virulence or load in determining the efficiency of mycobacterial eradication. A decrease in the CD4/CD8 ratio of macrophages is accompanied by a decrease in the proportion and total number of CD8 cells in the alveolar spaces can be a predictor for disease resolution in patients with active pulmonary tuberculosis.

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