Increased in CD8 T lymphocytes in the BAL fluid of patients with sulfur mustard gas-induced pulmonary fibrosis

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Summary

Objective: In an attempt to understand better the potential role of the T cell in the pathogenesis of pulmonary fibrosis (PF) due to sulfur mustard gas inhalation, this study was designed to analyze bronchoalveolar lavage (BAL) lymphocyte subsets and to determine the ratio of CD4 to CD8 lymphocytes in BAL fluid.

Setting: University hospital.

Patients: Twenty-one veterans with mustard gas-induced pulmonary fibrosis and 20 normal veterans as control group.

Intervention: Chest roentgenograms, pulmonary function tests (PFTs), tests for carbon monoxide diffusing capacity of the lung (DLCO), high-resolution CT scans of the chest, BAL via fiberoptic bronchoscopy, analyses of BAL fluids for cellular and Flow-cytometric analysis of the phenotype of bronchoalveolar cells were performed in all cases. A transbronchial lung biopsy was done in all patients following BAL.

Results: Neutrophilic alveolitis was the predominant feature. Neutrophils ($P < 0.0001$) and eosinophils ($P = 0.0006$) were the predominant cell types in the BAL fluid of patients with PF. CD8 lymphocytes expressed as percentage or absolute number were significantly higher in patients with PF than in healthy controls ($22.96 \pm 7.48\%$ vs. $14.16 \pm 7.73\%$, respectively; $P = 0.0006$; and $2.28 \pm 0.84$ vs. $1.10 \pm 0.55 \times 10^3$ cells/ml, respectively; $P < 0.0001$). The CD4/CD8 ratio was significantly lower in patients with PF than in healthy controls ($0.73 \pm 0.25$ vs. $1.58 \pm 0.67$; $P < 0.0001$). Except for the percentage and the absolute number of the BAL fluid neutrophils ($r = 0.70, P = 0.001$; $r = -0.62, P = 0.005$; respectively), no correlation was found between DLCO% and the other BAL cells. A significant negative correlation was observed between the percentage of DLCO and both the percentage and the absolute number of CD8 lymphocytes in BAL fluid in patients with PF ($r = -0.81, P = 0.0003$; $r = -0.61, P = 0.006$; respectively). A significant correlation was also seen between the percentage of DLCO and the CD4/CD8 ratio ($r = -0.60, P = 0.006$) in our patients.

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Introduction

The toxicity of the chemical warfare blistering agent sulfur mustard (2,2'-dichlorodiethyl sulfide; SM) has been investigated for nearly a century. This toxic gas can damage to the eyes and respiratory tract when present in high doses. Pulmonary fibrosis (PF) is a well-known late complication of sulfur mustard gas exposure in human. Although the respiratory tract lesions represent the major debility after sulfur mustard exposure, only a few studies have investigated the pathophysiology of sulfur mustard-induced respiratory diseases, in particular the mechanisms involved in inflammatory processes.

CD8 T cells infiltrate the lung in many clinical conditions, particularly in interstitial lung disease. The role(s) that CD8 T cells might be playing in the pathogenesis of inflammatory lung disease is unclear at present, as is the direct contribution of CD8 T cell effector activities to lung injury. According to this fact alterations in bronchoalveolar lavage (BAL) CD4/CD8 T cell subset ratios have been demonstrated in a variety of different respiratory disorders and the measurement of these changes may be diagnostically helpful. Therefore, the CD4/CD8 ratio in BAL is an index that is considered, at least in clinical respiratory medicine, as indicative for different types of interstitial lung disease. An elevated CD4/CD8 ratio in BAL is often seen in patients with sarcoidosis and other granuloma-producing diseases, such as asbestosis, Crohn’s disease, berylliosis, and rheumatoid arthritis. A reduced CD4/CD8 ratio has been observed in various other immunologic interstitial lung diseases, including drug-induced alveolitis, extrinsic allergic alveolitis, bronchiolitis obliterans organizing pneumonia (BOOP), and in patients infected with HIV. A normal CD4/CD8 BAL proportion has been described in patients with silicosis and asymptomatic granite workers.

It could, therefore, be anticipated that the lymphocytes may also play a role in the response to inhaled mustard gas. In an attempt to understand better the potential role of the T cell in the pathogenesis of PF due to sulfur mustard gas inhalation; this study was designed to analyze BAL lymphocyte subsets and to determine the ratio of CD4 to CD8 lymphocytes in BAL fluid in our victims with PF.

Methods

Patient population

Of all the veterans admitted to our university teaching hospital in 1986 with a single large exposure to sulfur mustard gas, 21 male patients were enrolled into this study after meeting all of the following criteria for PF diagnosis: (a) an interstitial pattern on a chest roentgenogram, (b) a restrictive pattern in spirometric studies, (c) a decreased single-breath carbon monoxide diffusing capacity of the lung (DLCO), and (d) a confirmatory transbronchial lung specimen biopsy specimen showing evidence of interstitial pneumonitis with varying degrees of interstitial fibrosis without evidence of granuloma formation. The patients’ exposure to sulfur mustard gas had been confirmed by studies on their urine and vesicular fluid in 1986.

None of the patients had any evidence of connective tissue disorders, sarcoidosis, eosinophilic granuloma, pneumoconiosis, carcinomatosis, or lymphoma. Patients with evidence of bacterial, mycobacterial and fungal infections were also excluded. All these patients had persistent intermittent cough from their exposures till now. Gradual decline in pulmonary function tests (PFTs) occurred following the initial gas exposure. The diagnosis of PF was made within 2 years of the acute and massive sulfur mustard gas inhalation in these victims. All were treated by intermittent low dose prednisolon (2.5–7.5 mg) for more than 6 years. All of the cases were lifetime nonsmokers. They signed a written informed consent form and underwent a thorough history and physical examination. Prior to undergoing bronchoscopy and PFTs, oral corticosteroid therapy was withdrawn for at least 1 month. A chest roentgenogram, an ECG, and a high-resolution CT (HRCT) of the chest were obtained in each patient. The HRCT scans were done with 1- to 1.5-mm sections taken at 1-cm intervals through the entire thorax and were reconstructed using a bone algorithm. The study was approved by the Ethics Committee of our university.

Control group

The control group consisted of 20 normal, healthy nonsmoking veterans with a mean (±so) age of 35.60±4.51 years old (range, 29–48 years old); the control subjects had participated in the Iran–Iraq War but had not been exposed to mustard gas. All subjects voluntarily entered the study and signed an informed consent form before their enrollment. All subjects had a complete history and physical examination. No subjects had a history of exposure to organic or inorganic dusts. In addition to obtaining a chest radiograph and an ECG for each subject, PFTs were measured prior to bronchoscopy as well. The examination of BAL cells and the determination of T cells were carried out for all cases using the same techniques as described for the patients group. No transbrachial biopsies were performed in the control group.

Measurement of pulmonary function and DLCO

Prior to bronchoscopy, PFTs were performed in all patients. These tests were measured through spirometric and lung volumes assessment according to the standards advocated.
by the American Thoracic Society. An experienced physician did all spirometric and lung volumes measurements by the helium dilution method with a Master Screen apparatus (Erich Jaeger GmbH, Wuerzburg, Germany), and DLCO by the single breathing handheld helium dilution method. Each patient was well trained to give his best effort. Results were expressed as percentage predicted based on accepted reference standards. The highest values were chosen and reported.

**Bronchoscopy and bronchoalveolar lavage (BAL)**

All of the patients underwent a bronchoscopic examination in 2005. Before any lung biopsy specimens were taken, BAL was performed using a flexible fiberoptic bronchoscope (Olympus BF1T; Tokyo, Japan). Each patient was premedicated with atropine (0.75 mg IM) before the procedure. Mild sedation was achieved with intravenously administered midazolam, and supplementary oxygen was given throughout the procedure. Patient oxygenation was monitored by pulse oximetry. After applying 4% lignocaine spray to the nose and throat of the patient, the flexible bronchoscope was introduced. Local anesthesia of the larynx was achieved with topical 4% lignocaine, whilst a 2% solution was used below the vocal cords to suppress coughing. The bronchoscope was wedged for lavage in the middle lobe segmental bronchus, and four 60-ml aliquots of sterile physiologic saline solution warmed to 37 °C were infused. The fluid was immediately recovered by gentle suction after each instillation. The first aliquot reflecting a bronchial sample was discarded, while the others were pooled for our study. The BAL fluid was passed through monolayer surgical gauze to eliminate mucus. One small aliquot of this fluid was utilized to count the total cell number, and another aliquot was spun to determine the cell count number. Another aliquot was spun in a cytometer at 400 revolutions/min for 10 min. The cell pellet was washed once in Hanks’ balanced salt solution (without calcium and magnesium). A May–Grunwald–Giemsa stain smear was used to identify the differential profiles after cytopsin preparation. Total cell counts were determined with a hemocytometer. The differential cell count of lymphocytes, neutrophils, macrophages, and eosinophils was made under light microscopy ×1000 by counting approximately 300 cells in a random field. The result was expressed as cells ×10³/ml. The unconcentrated supernatant was frozen at −70 °C before the protein was measured.

**Flow-cytometric analysis of the phenotype of bronchoalveolar cells**

To determine the T-lymphocyte phenotype, we employed immunofluorescent staining and flow cytometry. In order to identify the proportions of T lymphocytes, CD4, CD8 T cells, B cells and natural killer (NK) cells subpopulations of BAL fluid, cells were simultaneously stained with fluorescein isothiocyanate or (FITC) and phycoerythrin-conjugated (PE) monoclonal antibodies (CD3, -CD4, -CD8, -CD19, -CD56) (Beckon Dickinson, Mountain View, CA) according to the manufacturer’s protocol.

To determine the borderline between stained and unstained cells, cells were also stained with mouse IgG1 and IgG2a-conjugated FITC or PE. A cell gate containing lymphocytes was established on the basis of forward and side light scatter. Lymphocytes which were gated for CD3 expression, then further subdivided by CD4 or CD8 expression and analysed by two color flow cytometry on a FACScan. In other words, the relative ratio of CD4 or CD8 in CD3-positive cells was assayed by a dual-color analysis. Data were obtained and analyzed using Becton Dickinson BD LYSYS II and Cytometric Bead Array (CBA) software (San Jose, CA).

**Statistical analyses**

Data are presented as mean±SD unless otherwise stated. Comparisons were made using the Mann–Whitney nonparametric test for continuous data and by χ² analysis for discontinuous data. Correlations between different parameters were determined by Spearman’s rank correlation coefficient. P<0.05 was considered significant.

**Results**

**Demographics**

The characteristics of all study subjects are listed in Table 1. No control subject had an obstructive or restrictive pattern in his PFTs. The two groups were similar in age (P = 0.24).

**Chest radiography and high-resolution computed tomographic examinations**

On chest radiography (CXR), prominent reticular interstitial shadows, which are usually diffuse and patchy and most extensive in the lower zones, were seen in all cases. Bilateral abnormalities that may be asymmetrical are evident in all cases. However, overt honeycombing was not seen. CXR was normal in 2 cases. No nodules were seen.

The most common findings on HRCT were bilateral, reticular opacities with interlobular septal thickening c/w “fibrosis” (21 cases), ground-glass attenuation (10 cases), subpleural, bibasilar rim of increased attenuation (20 cases) and traction bronchiectasis (4 cases). Overt honeycomb changes were not seen.
cytes were seen when compared to the control group \( P \leq 0.04 \) than in the control group.

Among patients with PF than in healthy controls (22.96 vs. 8.25 positive cells, CD8 lymphocytes were significantly higher in the BALF of both the control and patients with PF are shown in Table 3. The profile of T-cell subtypes showed that when data were expressed as percentage or as absolute number of CD4 cells showed no significant diminishment in patients with PF compared to the control group (15.80 vs. 24.92 vs. 9.59; \( P = 0.0006 \)), while the absolute number of CD4 cells showed no significant difference between the two groups (1.57 vs. 9.0; \( P = 0.15 \)). Consequently, the CD4/CD8 ratio was significantly lower in patients with PF than in healthy controls (0.73 vs. 1.58 vs. 0.67; \( P < 0.0001 \)).

When CD3, B and NK alveolar lymphocytes were evaluated either as a percentage or as absolute number, no significant differences were found between the patients with PF and the control group (see Table 3).

### Comparison of the DLCO percentage with BAL fluid cellular constituents

Except for the percentage and the absolute number of the BAL fluid neutrophils \( (r = −0.70, P = 0.001; r = −0.62, P = 0.005; \) respectively), no correlation was found between DLCO\% and the other BAL cells.

### Comparison of the DLCO percentage with BAL lymphocyte subpopulations

A significant negative correlation was observed between the percentage of DLCO and both the percentage and the absolute number of CD8 lymphocytes in BAL fluid in patients with PF \( (r = −0.81, P = 0.0003; r = −0.61, P = 0.006; \) respectively) (Fig. 1). A significant correlation was also seen between the percentage of DLCO and the CD4/CD8 ratio \( (r = −0.60, P = 0.006) \) (Fig. 2) in our patients.

### Comparison of the parameters of pulmonary function with BAL lymphocyte subpopulations

BAL Lymphocyte subpopulations, whether expressed as a percentage or in cells per milliliter, had no significant correlations with the PFT physiologic parameters.

### Discussion

Respiratory disorders are the major cause of disability and mortality in mustard gas victims. There are several studies relevant to long-term respiratory disorders after mustard exposure in Iranian warfare victims.\(^4\) Its chronic pulmonary sequelae include airway narrowing or stricture, asthma, chronic bronchitis, bronchiectasis, and PF. Therefore, exposure to this gas should be added to the list of the various causes of interstitial lung diseases and PF.\(^22\)

In interstitial lung diseases, initial injury to epithelial cells is thought to be followed by alveolitis. The alveolitis is sometimes self-limiting, but may become progressive. In the latter case, lung fibrosis occurs. The mechanisms for these pathological changes are not fully understood. However,
Table 2  Comparison of bronchoalveolar lavage fluid cellular constituents of pulmonary fibrosis (PF) is due to sulfur mustard gas inhalation vs. the control group.*

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 20)</th>
<th>PF (n = 21)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery of BAL fluid (ml)</td>
<td>161.10±12.64</td>
<td>149.52±15.48</td>
<td>0.01</td>
</tr>
<tr>
<td>Number of cells (× 10^3/ml)</td>
<td>87.40±7.48</td>
<td>93.04±12.24</td>
<td>0.06</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>85.45±3.10</td>
<td>69.48±5.79</td>
<td>&lt;0.0001</td>
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<tr>
<td>Macrophages (× 10^3/m)</td>
<td>76.45±7.77</td>
<td>68.57±11.28</td>
<td>0.05</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>9.38±4.12</td>
<td>10.90±2.57</td>
<td>0.11</td>
</tr>
<tr>
<td>Lymphocytes (× 10^3/m)</td>
<td>8.25±3.71</td>
<td>10.04±2.33</td>
<td>0.06</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>2.49±1.32</td>
<td>13.25±3.94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Neutrophils (× 10^3/m)</td>
<td>2.15±1.08</td>
<td>12.19±3.68</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.64±0.71</td>
<td>2.33±1.68</td>
<td>0.0002</td>
</tr>
<tr>
<td>Eosinophils (× 10^3/m)</td>
<td>0.55±0.60</td>
<td>2.23±1.70</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

*Values are mean ± SD.

Table 3  Lymphocyte subpopulations in bronchoalveolar lavage from patients with PF due to mustard gas exposure and normal subjects.*

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 20)</th>
<th>PF (n = 21)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 cells (%)</td>
<td>44.90±10.32</td>
<td>42.30±7.08</td>
<td>0.17</td>
</tr>
<tr>
<td>CD3 cells (× 10^3 cells/ml)</td>
<td>3.60±1.66</td>
<td>4.19±0.98</td>
<td>0.35</td>
</tr>
<tr>
<td>CD4 cells (%)</td>
<td>24.92±9.59</td>
<td>15.80±5.61</td>
<td>0.0006</td>
</tr>
<tr>
<td>CD4 cells (× 10^3 cells/ml)</td>
<td>1.90±0.85</td>
<td>1.57±0.59</td>
<td>0.15</td>
</tr>
<tr>
<td>CD8 cells (%)</td>
<td>14.16±7.73</td>
<td>22.96±7.48</td>
<td>0.0006</td>
</tr>
<tr>
<td>CD8 cells (× 10^3 cells/ml)</td>
<td>1.10±0.55</td>
<td>2.28±0.84</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD4/CD8 (ratio)</td>
<td>1.58±0.67</td>
<td>0.73±0.25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B cells (%)</td>
<td>5.26±6.91</td>
<td>4.14±5.66</td>
<td>0.57</td>
</tr>
<tr>
<td>B cells (× 10^3 cells/ml)</td>
<td>0.50±0.60</td>
<td>0.38±0.49</td>
<td>0.49</td>
</tr>
<tr>
<td>NK cells (%)</td>
<td>2.91±6.65</td>
<td>1.46±3.74</td>
<td>0.39</td>
</tr>
<tr>
<td>NK cells (× 10^3 cells/ml)</td>
<td>0.20±0.41</td>
<td>0.38±0.49</td>
<td>0.63</td>
</tr>
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</table>

*Values are mean ± SD.

Figure 1  Relationship between the percentage of diffusion capacity for carbon monoxide (DLCO) and the percentage of CD8 T BAL cells in patients with pulmonary fibrosis (PF) due to mustard gas inhalation. There is a significant, negative correlation (P = 0.0003, r = 0.81) between the percentage of DLCO and the percentage of CD8 T BAL cells in PF.

Figure 2  Relationship between the percentage of diffusion capacity for carbon monoxide (DLCO) and the CD4/CD8 ratios in patients with pulmonary fibrosis (PF) due to mustard gas inhalation. A significant, positive correlation could be observed (r = −0.60, P = 0.006).
T-cells may have an important role in the pathophysiology of these diseases.15

The aim of this study was to characterize and compare BAL lymphocyte subpopulations in patients presenting with the clinical features of PF due to sulfur mustard gas inhalation and healthy subjects. To the best of the authors knowledge, this is the first study in which BAL lymphocyte subpopulations have been evaluated in these victims.

We demonstrated that patients with PF, when compared to healthy, were characterized by an increase eosinophils and neutrophils in BAL fluid. A positive correlation between either the percentage or the absolute number of BAL fluid neutrophils and percentage of DLCO from patients with PF has already been demonstrated.22 This study group corroborates our previous findings.22 These data also clearly demonstrate that the degree of lung function impairment closely related to neutrophilic alveolitis in patients with PF. It might act as a triggering factor in the development of PF in mustard gas-exposed patients.

The role of CD4+ or CD8+ T-cells in the regulation of PF has been investigated in several models of fibrosis.5,6,12,15

Analysis of T-lymphocyte subsets in BAL fluid indicates that, in patients with PF due to mustard gas exposure, CD4 T cells decrease and both the percentage and total numbers of CD8 T cells increase. This study on BAL fluid has also shown that the CD4/CD8 ratio is significantly decreased in patients with PF. We measured the relationship between T-lymphocyte subsets and physiologic indexes of pulmonary function tests in the patients with PF due to mustard gas inhalation. The percentage and the absolute number of CD8 T cells highly, negatively correlated with the percentage of DLCO (r = −0.81, P = 0.0003; r = −0.61, P = 0.006; respectively). As reported in other studies, the single breath DLCO is a very sensitive parameter for following-up deterioration and response to therapy in patients with idiopathic PF.23,24 Therefore, we present a set of observations that reveal that patients with more advanced lung function impairment as reflected by the DLCO, tended to reveal lower CD4/CD8 ratios.

Furthermore, it should be mentioned that the role of CD8 T-cells would be important in sulfur mustard gas-induced PF, since the CD8 population is greater than CD4 T-cell subset in the BAL fluid. The CD8 T-cell population could also be functionally divided into two subsets, Tc1 and Tc2, based on the cytokine secretion pattern.25 Recent reports disclosed that they may contribute to tumor rejection, protection against viral infection, and pathological changes in chronic obstructive pulmonary diseases.6,27 Therefore, it is possible that increased CD8 T cells in the BAL fluid may contribute to parenchymal destruction and therefore to the development of chronic fibrosis in these patients by releasing cytokines capable of increasing the susceptibility of target cells to cytotoxicity, or by secreting chemokines that attract other cells to the site of inflammation.28,29 The significant correlation observed in our veterans between increased number of CD8 T cells in the BAL fluid and reduced percentage of DLCO supports this hypothesis.

Although CD8 T cells are important effectors of cell-mediated immunity, their precise role in the pathogenesis of mustard gas-induced PF is unclear. An initial insult may lead to an influx of acute and chronic inflammatory cells that maintain immunological and inflammatory responses and thus produce progressive disease. It may be generally accepted that immune mechanisms are likely to make a contribution to pathogenesis; circumstantial support for an immunopathogenetic hypothesis includes the abundance of CD8 T-cells within the lung interstitium. Other inflammatory cells may be involved. Macrophages may produce a number of cytokines and neutrophils are likely to contribute to tissue damage by the generation of oxygen radicals and proteolytic enzymes.30 Eosinophils may contribute to lung injury through release of secretory products (eosinophilic cationic protein, vasoactive amines), cationic protein, vasoactive amines.31 Therefore, the end result of inflammatory and immunological events in mustard gas-induced PF is the presence of excess collagen within the lungs.

This analysis of BAL fluid from our patients with PF as a late sequel of massive sulfur mustard gas inhalation is similar to an experimental model of bleomycin-induced PF, it was found that the helper-to-suppressor ratio of T-cell lymphocytes shifted with time, having a value >1 at the beginning of the disease and a value <1 at the latest phases of disease.32 Overall, the BAL profile in our patients with PF due to sulfur mustard gas inhalation is characterized by several features: cell differentials with an increase in neutrophils, eosinophils and a decreased CD4/CD8 ratio. These features suggest this BAL profile pattern may be similar to the BAL fluid cellular constituents in usual interstitial pneumonia (UIP) but the finding of a decreased CD4/CD8 ratio (the absolute value) in the BAL fluid may be somewhat similar to cryptogenic organizing pneumonia (COP) or BOOP.33,34

In conclusion, CD8 T cells in BAL fluid were significantly elevated in patients with PF. Patients with higher grades of pulmonary fibrosis expressed as percentage of DLCO, revealed higher percentages and the absolute number of CD8 T cells and a lower CD4/CD8 ratio.

References


