Fibrosing alveolitis in systemic sclerosis: increase in memory T-cells in lung interstitium


ABSTRACT: Despite the large numbers of T-cells present in the lungs in fibrosing alveolitis, their pathogenetic role is poorly understood. If these cells are involved in pathogenesis, they are more likely to express the CD45RO+ memory phenotype. To test this hypothesis, open lung biopsies from patients with fibrosing alveolitis associated with systemic sclerosis (FASSc) were compared with grossly normal lung taken from the periphery of lobes resected for lung cancer.

Biopsies from eight patients with FASSc were compared with tissue from seven cancer controls. Paraffin sections were stained with a polyclonal anti-CD3 antibody for T-lymphocytes, monoclonal anti-CD45 antibody for leucocyte common antigen, and monoclonal anti-CD45RO antibody for primed T-lymphocytes. Staining was assessed quantitatively by computerized image analysis: in each case, the number of immunopositive cells was related to alveolar wall area and alveolar wall length.

Mean alveolar wall thickness was increased in patients with FASSc (60.7±24.0 μm) compared with cancer controls (15.7±5.3 μm). Patients with FASSc had greater numbers of CD45+, CD3+ and CD45RO+ cells·mm⁻¹ alveolar wall length compared with the controls. CD45RO+ cells made up 77% (median) of the CD3+ cells in FASSc, and their numbers per unit alveolar wall length were positively associated with alveolar wall thickness (r=0.61).

In conclusion, in fibrosing alveolitis of systemic sclerosis, most interstitial T-lymphocytes express the phenotype of memory cells; these cells are likely to be involved in the persistent inflammatory process.

Keywords: Fibrosing alveolitis memory T-cells systemic sclerosis

Accepted after revision November 18 1994

This study was supported by grants from the Raynaud's and Scleroderma Association, UK and The Scleroderma Federation and the United Scleroderma Foundation, USA.
Materials and methods

Patients

Open lung biopsies from eight patients (4 males and 4 females) meeting American Rheumatism Association criteria for a diagnosis of systemic sclerosis [9], who were also thought to have fibrosing alveolitis, were studied; the median age was 43 yrs (range 34–58 yrs) and two were smokers. Open lung biopsies were taken for diagnosis and staging of disease and not for research purposes. The clinical diagnosis of fibrosing alveolitis was confirmed histologically in all eight patients. Two patients were receiving penicillamine and one was receiving prednisolone (15 mg daily) and alpha-interferon. None was receiving azathioprine or cyclophosphamide. Biopsies were taken from the right lower lobe (RLL) (n=2), right middle lobe (RML) (n=2), right upper lobe (RUL) (n=2), left lower lobe (LLL) (n=1), left upper lobe (LUL) (n=1).

For comparison, samples of grossly and histologically normal peripheral lung were obtained from seven patients undergoing lobectomy for lung cancer: five males and two females, median age 71 yrs (range 35–74 yrs), of whom six were smokers. Samples were taken from peripheral uninvolved areas of the resected lobes.

Study Design

To evaluate morphological differences between FASSc patients and controls, mean numbers of immunopositive cells·mm\(^{-1}\) alveolar wall length were calculated for each of the three antibodies in each case. The numbers of CD3+ cells were also expressed as a percentage of CD45+ cells; numbers of UCHL1+ cells were expressed as a percentage of CD3+ cells. The mean numbers of immunopositive cells·mm\(^{-1}\) alveolar wall length were then compared between FASSc patients and controls. The relationship between alveolar wall thickness and the numbers of UCHL1+ cells, expressed per alveolar wall length and per alveolar wall area, was examined in 15 randomly selected fields in each of the eight patients with FASSc (i.e. a total of 120 fields).

Methods

Immunohistological examination of 10% neutral-buffered, formalin-fixed, paraffin-embedded 5 µm sections was performed following application of polyclonal anti-CD3 (T-cells, DAKO A452), monoclonal anti-UCHL1 (antigen-primed T-cells, DAKO M742) and monoclonal anti-CD45 (leucocyte common antigen, DAKO M701) antibodies, visualized using the alkaline phosphatase-anti-alkaline phosphatase method (APAAP) reported previously [10].

The number of positively stained cells was determined by observer-interactive computerized image analysis (Improvision-Apple Mac). Colour thresholding was used to highlight interstitial tissue (fig. 1a), and the area of alveolar wall was determined after alveoli, large blood vessels, bronchioles and lymphoid tissue had been excised by means of the "edit" facility. The alveolar wall outline was traced by the interactive "mouse" facility (fig. 1b). Numbers of immunopositive cells, alveolar wall length and alveolar wall thickness (area divided by length) were assessed for each field.

To determine the numbers of fields required to accurately estimate the mean for each section, numbers of CD3+ cells·mm\(^{-2}\) alveolar wall were tabulated for 10 randomly selected fields in sections from each of six patients. Estimation of the standard error of the mean within 95% confidence limits required a maximum of 63 fields, and within 90% confidence limits a maximum of 13 fields [11]; 90% confidence limits were chosen for the study. Formal scoring was then performed for each antibody in 15 randomly selected fields (objective ×10) in one section in each case.

Interobserver variability for two observers was assessed for the measurement of numbers of immunopositive cells (CD45RO+ cells), alveolar wall area and alveolar wall length in 15 randomly selected fields (five fields
Mean numbers of immunopositive cells per alveolar wall length were compared between FASSc patients and controls; nonparametric analysis was performed as the distribution of the mean numbers of immunopositive cells per unit alveolar wall length showed a positive skew. For analyses of correlation, the numbers of CD45RO+ cells per alveolar wall length were logarithmically transformed to normalize the distribution and the correlations between: 1) immunopositive cells per alveolar wall length and alveolar wall thickness; and 2) immunopositive cells per alveolar wall length and alveolar wall area were examined parametrically in 120 fields. A p-value of less than 0.05 was taken to be statistically significant.

Results

Histology of the biopsy specimens of patients with FASSc demonstrated a variable mixed pattern of mural and luminal inflammation and interstitial fibrosis. Some patients showed only mild mural thickening associated with accumulations of lymphocytes in areas of focal thickening and few alveolar macrophages (fig. 2a), whereas others showed marked accumulation of alveolar macrophages (fig. 2b). Several patients showed severe mural fibrosis with loss of alveolar architecture, bronchiolization of alveoli and hyperplasia of smooth muscle. Cystically dilated spaces lined by cuboidal alveolar lining cells were often present (fig. 2c). Vessel walls were often thickened and lymphoreticular aggregates were frequently found. Control tissue showed minimal thickening of alveolar walls (fig. 2d) and, in some cases, accumulation of alveolar macrophages containing tar bodies, in keeping with cigarette smoking.

Alveolar wall thickness

Mean alveolar wall thickness was greater in FASSc patients (60.7±24.0 µm) than in controls (15.7±5.3 µm) (p<0.001).

Immunohistochemical analysis

In patients with FASSc, significant increases were observed in the numbers of CD45+, CD3+ and CD45RO+ cells-mm⁻¹ alveolar wall length (fig. 3) (p<0.01), and cells-mm⁻² alveolar wall area (p<0.05). Immunopositive cells were widely distributed in the lung interstitium in FASSc patients (fig. 2a and b). The majority of cells
within lymphoid follicles (excluded from formal scoring) were CD3 and CD45RO negative; however, CD3+ and CD45RO+ cells on the edges of and immediately adjacent to lymphoid follicles were present in very high density (fig. 2c). The median value for the percentage of CD3+ cells which were CD45RO+ was 77% in systemic sclerosis compared with 36% in the control tissue. This difference did not, however, reach statistical significance (Wilcoxon rank sum test).

Numbers of primed T-cells in relation to alveolar wall thickness

Following log transformation to the base 10, the numbers of CD45RO+ cells·mm⁻¹ alveolar wall length, and cells·mm⁻² alveolar wall area, were evaluated in relation to alveolar wall thickness in each of the 120 fields scored in the eight patients with FASSc. There was a positive correlation between log number of CD45RO+ cells·mm⁻¹ alveolar wall length and the mean thickness of alveolar wall (r=0.61; p<0.001) (fig. 4a). However, when the number of CD45RO+ cells was related to the area of alveolar wall, the numbers remained constant with increasing alveolar wall thickness (fig. 4b), emphasizing that collagen deposition was not associated with a decline in T-cell numbers.

Discussion

The findings in the present study indicate that the interstitial infiltrate of mononuclear cells in FASSc consists largely of UCHL1 positive memory T-cells, suggesting that persistent inflammation involves cells which have an enhanced response to recall antigens which may be present locally within the lung, perpetuating the inflammatory response. Contrary to expectations, the numbers of memory T-cells per unit alveolar wall area did not decrease with the reduction in vascularity in regions of severe fibrosis, suggesting that there is a continuing signal favouring T-cell recruitment to the lung interstitium, even in advanced disease.

Immunocytochemistry has greatly facilitated the delineation of subpopulations of T-lymphocytes. It was initially assumed that surface markers defined distinct lineages of T-cells [12], but there is now much support for an alternative hypothesis proposing that selected surface markers define T-cells at different stages of maturation [13]. T-cell activation has been shown to result in a loss of CD45R positivity (defined by the presence of the 2H4 molecule) and a reciprocal gain in other markers, including CD29, CD2, lymphocyte function-associated antigens (LFA-1 and LFA-3), PGP-1 and CD45RO (UCHL1) [5, 14–16]; these distinct markers define the same broad subset of T-cells. The numbers of UCHL1+ and CD45RO+ cells are reciprocal; less than 1% of resting T-cells stain for both CD45R and UCHL1 [5]. Following antigenic re-exposure, proliferation is virtually confined to the UCHL1 + CD45R- subgroup [5]. On this basis, T-cells have been subdivided into
functionally "naive" uncommitted CD45R+ cells and "memory" (CD45RO+) cells which have been primed by antigen and are associated with marked responsiveness to antigen re-exposure [17–19]; this subdivision applies both to the CD4+ [20] and the CD8+ [19] T-cell subsets. This subdivision may not be so clear-cut, however, and there are now increasing data to suggest that isoform switching is not unidirectional, bringing into question the concept of "naive" and "memory" subset separation on the basis of CD45 expression [6, 7]. In normal volunteers, the great majority of T-cells obtained by bronchoalveolar lavage carry the CD45RO+ phenotype [21, 22], supporting the concept that the lung functions as an active immunological organ. Judging from studies of patients with chronic beryllium disease [22] and sarcoidosis [23, 24], the same preponderance of CD45RO+ T-cells is seen with bronchoalveolar lavage fluid in diffuse interstitial lung disease; the major difference between normal subjects and patients with chronic beryllium disease lay in the far greater absolute numbers of CD45RO+ T-cells in the latter [22]. The lack of correlation between bronchoalveolar lavage and interstitial cellularity complicates the interpretation of this data. This may also explain why the proportions of CD3+ T-cells which were CD45RO+ in our study were lower than reported in studies of lavage cells. Furthermore, this raises the interesting possibility that the evolution of CD45RO positivity occurs as the cells are recruited to the epithelial lining fluid during their transition through the interstitium. Despite these quantitative differences, our findings demonstrate that the same absolute increases in CD45RO+ and other immunopositive cells, without major changes in their relative proportions are seen within lung interstitium in fibrosing alveolitis.

The intense infiltration of lung interstitium by memory T-cells in the present study suggests that antigenic stimulation of T-cells may have a role in the ongoing chronic inflammation of FASSc, and is consistent with studies in rheumatoid arthritis and multiple sclerosis, two autoimmune diseases in which an increase in the proportion of T-lymphocytes which are memory T-cells has been observed [25, 26]. Studies of the T-cell antigen receptor repertoire would be needed to determine if this is an oligoclonal response, as would be expected if the T-cell response was driven by specific antigen(s).

In the present study, the numbers of memory T-cells per unit alveolar wall length increased in proportion to alveolar wall thickness, and, thus, T-cell numbers per unit alveolar wall area remained constant. This finding, applied to fields in which alveolar walls were markedly thickened by fibrosis, emphasizes that T-cell accumulation occurred even in advanced disease. If T-cell responses wane as fibrosis develops, their numbers might be expected to decline in areas of marked alveolar wall thickening, in keeping with the concomitant reduction of capillaries. The fact that memory T-cell numbers are maintained, despite a short lifespan in vivo [27] and a reduction in blood supply in severely affected regions of the lung [28], suggests that they are actively recruited, even to areas of marked fibrosis. The mechanism of recruitment and the subsequent relationship of T-cells to lung fibroblast proliferation and collagen deposition following antigen-priming and activation requires further study. One possibility is that T-cells play a role in regulating the deposition of collagen, a concept for which there is recent support from animal studies [29].

In the present study, a computerized image analysis programme was employed to quantify cell numbers, alveolar wall area and alveolar wall thickness. This method of quantification of inflammatory cell numbers in relation to alveolar wall thickness and length is time-consuming but has some advantages over semi-quantitative scoring systems evaluated and employed in earlier work [30–33]; whilst semi-quantitative systems are suitable for making observations of major differences between patient groups, they offer less precision and reproducibility.

In a recent study of interobserver variability among experienced histopathologists, the kappa coefficients of agreement varied from -0.06 to 0.30; for most variables, agreement between observers in assigning a semi-quantitative grade was little higher than that expected by chance [30]. In another study, an eight point scale was constructed to quantify the degree of pulmonary fibrosis; however, interobserver variability was substantial (coefficient of variation greater than 30%) [31]. In the present study, interobserver variability in the measurement of alveolar wall length and alveolar wall area was minimal (coefficients of variation less than 5%). The interobserver variability associated with measurement of the numbers of immunopositive cells was higher than hoped (coefficient of variation 12%) and was largely attributable to the selection of the colour cut-off used to define immunopositivity. This limitation should be borne in mind in studies demonstrating subtle differences between subgroups; however, interobserver variability was considerably lower than the differences observed between subgroups.

In conclusion, we report that most T-cells in the lung interstitium of patients with FASSc are of the memory phenotype: the numbers of these cells are maintained in the areas of greatest alveolar wall thickness, despite the associated reduction in blood supply. These findings suggest that T-lymphocytes are actively recruited to the lung, and are likely to play a part in maintaining chronic inflammation and contributing to the slow but progressive deterioration of lung function in systemic sclerosis.

References

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