Gamma/delta cells in tissue from patients with sarcoidosis

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Abstract

Background – Because gamma/delta T lymphocytes ($\gamma\delta$ cells) respond to mycobacterial antigens in vitro and accumulate in the skin lesions of patients with certain granulomatous infections (leprosy, leishmaniasis), it was hypothesised that these cells might have a role in the pathogenesis of sarcoidosis, a disease also characterised by granuloma formation. Having failed to demonstrate an increase in $\gamma\delta$ cells in the blood of patients with sarcoidosis, the aim of this study was to examine samples of bronchoalveolar lavage (BAL) fluid and biopsy tissue.

Methods – Samples from 23 patients (13 women) with newly diagnosed sarcoidosis, of mean age 31 years and median percentage of lymphocytes in the BAL fluid of 31%, were studied. Controls included normal subjects and patients with other interstitial lung diseases (ILD). Cytopreparations of BAL fluid (n=13) and cryostat sections (five mediastinal nodes, 14 transbronchial biopsies) were stained with alkaline phosphatase-antialkaline phosphatase and monoclonal antibodies to CD3, CD4, CD8, CD25, and $\gamma\delta$ T cell receptor (TCR).

Results – All patients had typical chest radiographs (16 stage I, four stage II, three stage III). All were Mantoux negative with negative tuberculosis cultures. Compared with normal controls and patients with other interstitial lung diseases there was no increase in $\gamma\delta$ cells in the BAL fluid (sarcoidosis, 1% (range 0–4%) total cells; ILD, 1% (0–2%); controls, 0.5% (0–2%); $p>0.05$, Kruskal-Wallis). Likewise, there was no increase in $\gamma\delta$ cells in the transbronchial biopsy specimens (sarcoidosis, 1/high power field (hpf) (range 0–2); ILD, <1 /hpf (0–4); controls <1 /hpf (0–2); $p>0.05$). $\gamma\delta$ cells were rarely seen in the lymph nodes in spite of the presence of numerous granulomas.

Conclusion – These results provide further evidence that $\gamma\delta$ cells are not increased in most patients with sarcoidosis.

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these cells recognise a variety of mycobacterial antigens in vitro and accumulate in the granulomatous skin lesions of leprosy and cutaneous leishmaniasis.⁴ In murine models $\gamma\delta$ cells tend to accumulate at epithelial surfaces such as the skin, gut and lung, suggesting that they represent the first line of immune defence as they are found at sites of initial antigen contact.^{5–7}

Although the aetiology of sarcoidosis remains unknown, evidence points to an antigen driven T cell response with cytokine production and subsequent granuloma formation.^{8,9} Because $\gamma\delta$ cells are associated with infectious granulomatous responses, we asked whether these cells might also have a role in the pathogenesis of sarcoid granuloma. We have previously found no increase in the mean percentage or absolute numbers of $\gamma\delta$ cells in the peripheral blood of 21 patients with newly diagnosed sarcoidosis, although two patients had increased proportions of $\gamma\delta$ cells.¹⁰ These findings have been supported by other publications.^{11–14}

Although a number of studies have reported a variable proportion of patients with increased $\gamma\delta$ cells in bronchoalveolar lavage (BAL) fluid,^{13–15} only two groups have examined biopsy material from patients with sarcoidosis. Nakata *et al* examined lung biopsy specimens from five patients and found few $\gamma\delta$ cells.¹⁶ Tazi *et al* examined node and Kveim biopsy tissues and failed to find any increase in $\gamma\delta$ cells.¹⁷

In this study we sought to extend our initial findings by looking for $\gamma\delta$ cells in tissue samples from patients with newly diagnosed untreated sarcoidosis.

Methods

SUBJECTS AND CONTROLS

Samples were obtained from 23 patients (13 women) of mean age 31 years with newly diagnosed sarcoidosis who were on no treatment at the time of diagnosis or within the previous year (table 1). The diagnosis was established using previously described criteria and all but two patients had non-caseating granulomas in the biopsy samples.¹⁸ These two patients presented with erythema nodosum and hilar lymphadenopathy which resolved spontaneously and both had a BAL lymphocytosis with negative screen for tuberculosis. Eight patients were smokers and all were Mantoux negative, 22 of the 23 having previously received BCG vaccination. The median BAL lymphocyte percentage was 31% (range 4–60) and 14 patients had an absolute blood lymphopenia (<1.5 cells/ μ l). The chest radiograph was abnormal in all cases: 16 stage I; four stage

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T cells expressing gamma/delta ($\gamma\delta$) T cell receptor (TCR) have been postulated as having a role in the pathogenesis of several infectious disorders characterised by the formation of granuloma at sites of infection.^{1–3} In humans

Table 1 Patient characteristics

	Sarcoidosis (n = 23)	Control† (n = 12)	Other ILD* (n = 13)
Mean age (years)	31	45	49
M:F	10:13	8:4	5:8
Previous BCG	22	5	3
Positive Mantoux	0	1	0
Abnormal chest radiograph	23 (stage I, 16) (stage II, 4) (stage III, 3)	6	13
Median (range) % lymphocytes in BAL fluid	31 (4–60)	4 (1–8)	18 (2–34)
Samples stained			
BAL fluid	13	6	9
Transbronchial tissue	14		6
Mediastinal node	5		
Thoracotomy lung		6	

* Other interstitial lung diseases included EAA (2), pulmonary fibrosis (8), lymphangitis (1), pneumonitis (1), lymphangio-leiomyomatosis (1).

† Controls included patients with cough undergoing bronchoscopy or patients undergoing lung resection for benign or malignant tumour.

II; three stage III. BAL samples were available for study from 13 patients, transbronchial biopsy specimens from 14, and mediastinal lymph nodes from five.

Normal controls included patients undergoing bronchoscopy for cough or minor haemoptysis in whom the chest radiograph was normal and no bronchoscopic abnormality was present (n = 6), and patients undergoing thoracotomy for lung cancer in whom tissue for study was taken away from the affected area (n = 6). Patients with other interstitial lung diseases (ILD) were used as further controls (n = 13) (table 1).

TISSUE SAMPLES

Bronchoalveolar lavage was performed using standard techniques. Briefly, 180 ml was instilled into the lateral basal segment of the right lower lobe and aspirated using gentle suction. The specimen was transported on ice and cytopreparations were made for Diffquick staining (for cell differential counting) and monoclonal antibody labelling. Cytopreparations were frozen at -20°C prior to immunohistochemical analysis.

Transbronchial biopsy specimens and thoracotomy lung tissue samples were mounted in OCT and snap frozen in isopentane prior to storage at -70°C . Mediastinal lymph node biopsy specimens were snap frozen and stored in liquid nitrogen. Cryostat sections ($5\mu\text{m}$) from all samples were taken for immunohistochemical analysis.

IMMUNOHISTOCHEMICAL ANALYSIS

Monoclonal antibodies to the following antigens were used as the primary antibody: CD3, CD4, CD8, CD25, and $\gamma\delta$ (TCR $\delta 1$ recognising the δ protein expressed in association with the γ chain and hence all $\gamma\delta$ positive cells). Controls included cells incubated without the primary antibody or isotype matched antibody added instead. Sections of human tonsil were used as positive controls for CD3 positive α/β cells, and blood and sections of skin from a patient with intestinal lymphangiectasia were used as positive controls for CD3 positive $\gamma\delta$ cells.

Table 2 Immunocytochemical analysis of cytopreparations of BAL fluid

Antigen	Sarcoidosis	Interstitial lung disease	Normal control
$\gamma\delta$	0 (0–0.3)	0 (0–1)	0 (0–0.1)
CD3	31** (6–60)	26** (7–63)	6 (2–10)
$\gamma\delta/\text{CD3}$	0 (0–4.0)	0 (0–4.2)	0 (0–1)
CD4	19* (2–32)	17* (9–35)	3 (0.5–6)
CD8	5* (1–17)	13* (2–24)	1 (0.6–4)
CD25	0.6 (0–4)	0.3 (0–3)	0 (0–0.1)

Data are presented as percentage positively staining cells of total BAL fluid cells with $\gamma\delta$ cells also expressed as a percentage of CD3 positive cells. Values are median (range).

* $p < 0.05$, ** $p < 0.01$, Kruskal Wallis one way analysis of variance.

For light microscopy BAL cytopreparations were stained with Diffquick and histological specimens were fixed in formalin and stained with haematoxylin and eosin. For immunohistochemistry all slides were fixed with methanol/alcohol and thoroughly dried prior to incubation with monoclonal antibodies.¹⁹ Briefly, the alkaline phosphatase-antialkaline phosphatase (APAAP) technique (Dako) was used; the slides were incubated with 20% AB serum for 20 minutes before adding the primary antibody for 30 minutes, washed and then incubated with the secondary antibody (alkaline phosphatase rabbit anti-mouse) for 30 minutes. After washing again the slides were incubated with APAAP for 30 minutes, washed, and steps 2 and 3 repeated to amplify the reaction prior to staining with the substrate (levamisole/fast red). The slides were then counterstained with Mayer's haematoxylin (blue) and mounted in glycerol/gelatin.

DATA ANALYSIS

A minimum of 300 cells was counted for each BAL cytopreparation and the mean number of positively staining cells expressed as a percentage of the total cells in the BAL fluid. A minimum of three high power fields (magnification $\times 400$) was examined for each cryostat section. All slides were examined by two observers blinded to the clinical diagnosis. The coefficient of variation for inter-observer and intra-observer observations was less than 15%. Because of the small size of the biopsy specimens and the low number of positively staining cells, a grid was not routinely applied. For the same reason $\gamma\delta$ cells are expressed as absolute numbers per high power field (hpf) as well as a derived percentage of CD3 positive cells.

Results were analysed using the Kruskal-Wallis one way analysis of variance. Differences were considered to be significant at the 5% level. Data are expressed as median plus range.

Results

BRONCHOALVEOLAR LAVAGE FLUID

Compared with samples from normal controls and those with other interstitial lung diseases, there was no increase in $\gamma\delta$ cells in the samples of BAL fluid (table 2). Both patient groups had more CD3 positive cells than normal controls ($p < 0.01$, Kruskal Wallis) and corresponding significant increases in CD4 and CD8 positive

Table 3 Immunohistochemical analysis of lung biopsy samples

Antigen	Sarcoidosis	Interstitial lung disease	Normal control
$\gamma\delta$	0.2 (0-4)	0.2 (0-1)	0.55 (0-3)
CD3	21 (3-30)	23 (8-37)	23 (6-25)
$\gamma\delta$ /CD3	1.8 (0-18)	1.2 (0-11)	2.9 (0-4.6)
CD4	13 (2-31)	14 (10-23)	15 (3-21)
CD8	8 (2-22)	11 (6-21)	9 (3-25)
CD25	0.8 (0-2)	1.1 (0.5-2)	0.2 (0-2)

Data are presented as positively staining cells per high power field with $\gamma\delta$ cells also expressed as a percentage of CD3 positive cells. Values are median (range).

There were no significant differences between the groups (Kruskal Wallis one way analysis of variance).

cells ($p < 0.05$). Although the percentage of CD25 positive cells was higher in the subjects with sarcoidosis and other interstitial lung diseases, because of the small numbers of positively staining cells this did not reach statistical significance.

TRANSBRONCHIAL BIOPSY SPECIMENS

The patients with sarcoidosis had scant numbers of $\gamma\delta$ cells in the biopsy specimens regardless of whether or not granulomas were present. There was no significant difference in the percentage of $\gamma\delta$ /CD3 positive cells between the three groups (table 3).

MEDIASTINAL LYMPH NODES

In all five lymph nodes numerous granulomas were seen with more than 100 CD3 positive cells/hpf. $\gamma\delta$ cells were rarely seen and constituted less than 1/hpf and, even as a derived percentage of CD3 positive cells, less than 1%.

Discussion

We have shown in this study that patients with newly diagnosed sarcoidosis do not have increased numbers of $\gamma\delta$ cells in their BAL fluid, transbronchial biopsy or lymph node biopsy specimens. These results are in accord with our earlier published results.¹⁰ We have endeavoured in this study to recruit patients with active disease as evidenced by symptoms such as fatigue or erythema nodosum and clinical findings such as peripheral blood lymphopenia and BAL lymphocytosis. None of our patients had any evidence of tuberculous infection or disease which might have confounded our results, as it has been reported that $\gamma\delta$ cells are increased in tuberculosis.²⁰

We examined the specimens of BAL fluid using cytopreparations and APAAP staining in order to minimise the confounding effects of autofluorescence of the alveolar macrophages during fluorescence activated cell sorter (FACS) analysis. Our initial studies used FACS exclusively to assess staining of BAL fluid and, although the results were similar to those obtained with the APAAP method, there was considerable variability. Adhering the macrophages out has been suggested as a way to reduce the problem of autofluorescence in BAL samples, but $\gamma\delta$ cells themselves can be ad-

herent and this technique could have led to bias (unpublished observation).

Our results in this study are similar to previously published reports. Forrester *et al* found only a small percentage of $\gamma\delta$ cells in the BAL fluid.¹³ Balbi *et al* showed that the percentage of $\gamma\delta$ cells in the BAL fluid of nine patients was similar to that in their blood and to the blood of normal control subjects.¹⁴ However, in an earlier paper Balbi and other authors found increased numbers of CD3 positive $\alpha\beta$ negative cells (hence presumptive $\gamma\delta$ positive) in patients with sarcoidosis.¹¹ More recently, Raulf *et al* found higher numbers of $\gamma\delta$ cells in BAL fluid from patients with sarcoidosis compared with idiopathic pulmonary fibrosis.¹⁵ Nakata *et al* did not find increased numbers of $\gamma\delta$ cells in the BAL fluid and specifically noted that three patients with high proportions of $\gamma\delta$ cells in the blood had low numbers of these cells in their BAL fluid.¹⁶ These same authors studied lung biopsy tissue from five patients and reported small numbers of $\gamma\delta$ cells present (0-2.3% CD3 positive cells). Tazi *et al* examined lymph nodes from four patients and Kveim biopsy specimens from four and observed only rare $\gamma\delta$ cells inside or at the periphery of granulomas.¹⁷ Our findings are identical.

Although these studies viewed collectively suggest that few patients with sarcoidosis have an increased number of $\gamma\delta$ cells in the BAL fluid, it has been shown that a higher proportion of these cells express V δ 1 gene segments.¹³ This finding is not universal, however, as two other studies have shown that some patients with sarcoidosis have increased V δ 2 transcripts or cells staining positively with anti-TCR V δ 2.^{14,21} In spite of our findings, it is still possible that $\gamma\delta$ cells may be important for some patients with this disease, particularly those of Asian or Middle Eastern origin.²² However, the failure to find increased numbers of $\gamma\delta$ cells in association with granulomas suggests that, for most patients, $\gamma\delta$ cells are not important in the pathogenesis of sarcoidosis.

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