Relation between immunocytological features of bronchoalveolar lavage fluid and clinical indices in sarcoidosis

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ABSTRACT This study was designed to determine whether cell populations in bronchoalveolar lavage fluid represent a reflection of disease activity in sarcoidosis. Bronchoalveolar lavage fluid cells were obtained from 22 patients with sarcoidosis and from 10 normal control subjects and investigated by immunocytological methods. A panel of monoclonal antibodies was used to determine the relative proportions of phenotypically distinct subsets of macrophages and lymphocytes in the patients with sarcoidosis and to correlate them with clinical indices, such as disease duration, serum angiotensin converting enzyme, the chest radiograph, and results of pulmonary function tests. Patients with sarcoidosis had a higher percentage than the normal subjects of macrophage-like cells expressing RFD1 (a class II associated antigen preferentially expressed by dendritic cells), an epithelioid cell antigen (RF9D), and a circulating monocyte antigen (UCHMI). The increase in RFD1+ cells appeared to be due to detection of antigen by this antibody on cells that were also expressing phenotypic markers of classical tissue macrophages (RF7). The lymphocytes in lavage fluid from patients with sarcoidosis were characterised by increased expression of activation markers, such as interleukin-2 receptors (anti-Tac+), HLA-DR (RFDR+), and “blast” forms (expressing above normal concentrations of CD7 antigen). This was associated with increased proportions of the CD4+ (helper-inducer) T cell subset. Patients with sarcoidosis whose clinical indices suggested activity showed an increased number of macrophages coexpressing RFD1 and RF7 antigens, of macrophages expressing UCHM1 and lymphocytes expressing activation markers. The expression of these markers was also increased on lavage cells from patients with radiographic evidence of widespread disease (chest radiographic stage II and III), but there was no relation with disease duration, pulmonary function, or serum angiotensin converting enzyme activity. Immunocytological analysis of lavage cells offers a probe for studying the pathogenesis of sarcoidosis and may be of value in monitoring disease activity.

Introduction

Alveolar macrophages and lymphocytes have an important role in the immune mechanisms underlying sarcoidosis.12 Previous studies have shown that alveolar macrophages are extremely heterogeneous and no clear difference has been seen between patients with different types of interstitial lung disease and normal control subjects in the proportions of cells staining positively with different monoclonal antibodies.13

The aim of our study was to use a panel of monoclonal antibodies to try to correlate cell membrane markers with disease activity and other clinical indices in sarcoidosis. Several studies have attempted to identify indices that correlate with disease activity and could be used to predict or monitor its course. Inconclusive data have accumulated from studies looking at differential cell counts,6-11 or lymphocyte subsets in bronchoalveolar lavage fluid,11,12 gallium scans,6-10,12 and serum angiotensin converting enzyme activity.6,10,12-14 The initial promise that a “high intensity alveolitis,”13 defined as more than 28% T lymphocytes in the lavage fluid differential count plus a high gallium score, predicted disease activity and clinical deterioration has been tempered by conflicting results from other series.6,7,16,17 Recently Turner-Warwick et al have suggested that a high quality chest radiograph together with pulmonary function tests

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were as good as any of the more sophisticated, expensive, and invasive tests in monitoring disease progression. The clinical value of bronchoalveolar lavage, or at least of the differential cell count, which is the test most commonly used with lavage fluid, has yet to be proved.20

Immunocytological analysis of macrophages and lymphocytes in bronchoalveolar lavage fluid might provide a useful tool to investigate the pathogenetic mechanisms concerned in the initiation and progression of sarcoidosis, and could be of clinical value in monitoring disease activity.

Methods

STUDY POPULATIONS

Control subjects

We studied seven normal volunteers (six male, one female; age 22–41 years) and three patients (one female, two male; age 32–70 years) who were having fibreoptic bronchoscopy for investigation of a single episode of haemoptysis (chest radiograph normal) or a localised mass lesion (bronchoalveolar lavage performed on the normal lung). Three (one volunteer, two patients) were current cigarette smokers; none was receiving medication.

Patients with sarcoidosis

The patients with sarcoidosis consisted of 11 women and 11 men (age 26–76 years) with histological evidence of non-caseating granulomas in transbronchial lung biopsy specimens, in the appropriate clinical setting and in the absence of other known causes of pulmonary granulomatous disease. Eight patients were ex-smokers (five having stopped more than a year before lavage), and three smoked less than five cigarettes a day. Disease duration varied from three months to 12 years (mean 2.3 years). One patient had a stage 0 chest radiograph (that is, normal), five had stage I (clear lung fields but bilateral hilar lymphadenopathy), 11 had stage II (bilateral hilar lymphadenopathy and lung field shadowing), and five had stage III (lung field shadowing but no hilar adenopathy). Only one of these five had had enlarged hilar nodes noted previously. Eleven (50%) had a forced vital capacity (FVC) or transfer factor for carbon monoxide (TLCO), or both, less than 80% of predicted normal values. Fourteen had abnormally raised serum angiotensin converting enzyme activity. Seven patients had evidence of extrathoracic disease (peripheral lymphadenopathy in three, hepatomegaly, myopathy and peripheral lymphadenopathy in one, and hepatomegaly and nasal and skin lesions in one each). No patient was receiving treatment at the time of lavage; eleven subsequently started taking corticosteroids because of symptoms, deteriorating pulmonary function, or persistent or increasing shadowing on the chest radiograph. This was instituted by the doctor who was looking after the patient, who had no knowledge of our assessment of disease activity or immunocytology. Table 1 shows clinical details of the patients at the time of bronchoalveolar lavage.

CLINICAL SCORE

We used the scoring system shown in table 2 to assess clinical activity in each patient at the time of bronchoalveolar lavage, and again at follow up 6–12 months later. This gives a maximum score of 10 points; arbitrarily, any patient with a score of 5 or more was judged to have active disease. Similar systems have been used in other studies.14223 The maximum mark for radiographic abnormality was 3/10 and for pulmonary function abnormality 4/10, so that neither could influence the total score unduly. The International Labour Office (ILO) chest radiograph scoring system was used in addition to staging so that the profusion of parenchymal shadowing could be measured in patients with lung field shadowing. We found that giving different weightings to the various components of the score failed to alter the total significantly.

On the basis of the initial score and the 6–12 months follow up 15 patients were judged to have active disease and seven inactive disease at the time of the bronchoalveolar lavage. None of the seven patients judged to have inactive disease developed active disease subsequently either as judged by score or in the opinion of the doctors looking after them. Of the 11 treated patients with active disease who received treatment, seven showed partial and four complete resolution. Of the four untreated patients who received no treatment two showed partial and two complete resolution. This suggests that a high score represents active disease rather than “burnt out” severe disease, in that the 15 patients judged to have active disease showed a substantial decrease in scores over the follow up period, with and without treatment (table 1).

BRONCHOALVEOLAR LAVAGE

Approval for the study was given by the hospital ethics committee and informed consent was obtained from all patients and volunteers.

Premedication was given one hour before the procedure and consisted of 10 mg intramuscular papaveretum and 0-6 mg atropine. The upper respiratory tract was anaesthetised with topical 4% xylocaine. An Olympus BFITR fibroscopic bronchoscope was introduced transnasally into the lower respiratory tract, where 2% xylocaine was sprayed. The instrument was then wedged in a lateral subsegmental bronchus in either the right middle or the right lower lobe of the supine patient. A total of 180–240 ml of sterile 0-9% normal saline (corrected to pH 7.4 with...
Relation between immunocytological features of bronchoalveolar fluid and clinical indices in sarcoidosis

Table 1  Clinical details of patients with sarcoidosis

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*Other than caucasian.
†Classification: 0—clear radiograph; 1—bilateral hilar lymphadenopathy; II—bilateral hilar lymphadenopathy and lung field shadowing; III—lung field shadowing only.
LN—lymph node; FVC—forced vital capacity; TLCO—transfer factor for carbon monoxide; SACE—serum angiotensin converting enzyme activity in nmol/ml/min (normal range 15–53); BAL—bronchoalveolar lavage.
‡See under "Methods."

8.4% sodium bicarbonate and maintained at 37°C was introduced in 60 ml aliquots and removed by gentle suction. The fluid was collected into a sterile siliconised glass bottle at 4°C.

PROCESSING THE LAVAGE FLUID

The total cell count was determined from an aliquot of neat unfiltered lavage fluid in a modified Neubauer haemocytometer. Viability of the cells was assessed in all cases by trypan blue exclusion and was always greater than 70%. Mucus strands were removed by filtering the fluid through a layer of sterile gauze. The fluid was then centrifuged at 450 g for 10 minutes and the supernatant was decanted from the cell pellet. The cells were then washed twice with Hanks’s balanced salt solution (HBSS) and the cell suspension was adjusted to a concentration of 2–3 × 10⁶ cells/ml. Cytospin slide preparations were obtained with 50–100 µl aliquots in a Shandon Cytospin II (Shandon Instruments, Runcorn). The cytospin slides were then air dried for one to two hours and fixed in a 1:1 chloroform:acetone mixture for 10 minutes. They were then air dried again for one to two hours and stored (wrapped in plastic cling film) at −20°C until use (from one week to 2 years). Differential counts were performed immediately on cytospin preparations stained with May-Giemsa-Griinwald, a total of 300–600 cells being counted.

IMMUNOCYTOLICAL ANALYSIS

The panel of monoclonal antibodies used in the study is shown in table 3. Their pattern of reactivity to cells in normal tissues has been characterised. RFD1* and RFD7* appear to identify distinct macrophage subsets in normal tissues.

IMMUNOPEROXIDASE STAINING

A standard protocol for the indirect immunoperoxidase method was used. The cytospin preparations were first incubated with each monoclonal antibody and phosphate buffered saline (PBS), at an optimal dilution, for one hour. After washing in PBS, the cytospin preparations were incubated for 45 minutes
with peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO Immunoglobulin a/s, Copenhagen). After further washing in PBS the preparations were developed for 5–15 minutes in freshly made dianisobenzidine and hydrogen peroxide solution. The cytopsins were counterstained in haematoxylin for 20–40 seconds, dehydrated, mounted with coverslips in DPX, and examined under a light, oil immersion microscope. Three hundred cells were counted in successive high power fields and assessed as being either positive or negative (background staining only) for each antibody; the results were expressed as the percentage of cells that were positive. Positive controls (tonsil sections) were used with each antibody in all staining sessions. Negative controls (PBS incubation instead of first layer monoclonal antibody) were used for each patient, to assess background staining.

DOUBLE IMMUNOFLOURESCENCE
A standard technique for the simultaneous identification of two cellular antigens was used. Fifty microlitres of each relevant monoclonal antibody were mixed and incubated with the cytopsin preparations for one to two hours. After being washed in PBS, they had a 50 μl mixture of goat anti-mouse immunoglobulin M tetraethyl rhodamine isothiocyanate (TRITC) and goat anti-mouse immunoglobulin G fluorescein isothiocyanate (FITC) (both from Southern Biotechnology Associates) added as a second layer; this was incubated for 45 minutes. After the PBS washing, the cytopsin slides were mounted with coverslips in PBS and glycerol and examined immediately with a fluorescence microscope. Two hundred cells were counted in successive high power fields, first under phase contrast and then with appropriate barrier filters for TRITC and FITC. The cells fluorescing either red only, green only, or both were counted and the number was expressed as a percentage of the total cells. Again positive and negative controls were always performed.

ANALYSIS
Statistical analysis was performed with Student's t test or, when the results were not normally distributed, the Mann-Whitney U test.

RESULTS

DIFFERENTIAL CELL COUNTS IN LAVAGE FLUID
The differential cell counts in lavage fluid in the normal control subjects and patients with sarcoidosis are shown in figure 1. Total cell counts were higher in the patients with sarcoidosis than in the control subjects (p < 0.005). The mean (SEM) percentage of lymphocytes was higher (34 (3)) and the proportion of macrophages lower (61 (4)) than the values in the normal subjects (73 (3) and 91 (3) respectively). The total numbers of both macrophage like cells and lymphocytes were higher in the patients with sarcoidosis (p < 0.01).

IMMUNOCYTOLOGICAL ANALYSIS

Macrophage like cells

Virtually all the lavage cells with macrophage morphology from both the normal subjects and the patients with sarcoidosis expressed RF–DR (mean 86% (SEM 2%) and 92% (2%) respectively, fig 2a). The percentage of the cells that were RFDI* was significantly higher in the patients with sarcoidosis (69 (5)) than the normal subjects (39 (5), p < 0.001), whereas the percentage of RDFD* cells did not differ significantly between the two groups (33 (4)).
sarcoid (45 (4); p > 0.05). Only a small percentage of macrophage like cells in lavage fluid from normal subjects expressed RFD1 and RFD7 (10 (2)) with double immunofluorescence, whereas a substantial percentage of cells (36 (3)) in the samples from patients with sarcoid were RFD1+ and RFD7+ (p < 0.0001). In lavage fluid from normal subjects only a small percentage of cells were RFD9+ (9 (1.5)) or UCHM1+ (7 (3)); in patients with sarcoidosis the percentages of both were higher (29 (4) and 28 (5) respectively; p < 0.01). There was no significant difference in the proportion of macrophages expressing any of these markers between smokers and non-smokers in either group. There was also no difference between the results from the control patients and the normal volunteers.

LYMPHOCYTES
No identifiable B cells were found in any sample (data not shown). Of the T cells, CD4+ cells (T mix+, CD8- cells, helper-inducer lymphocyte subset) outnumbered CD8+ (suppressor-cytotoxic lymphocyte subset) cells in all samples. The ratio CD4:CD8 was higher in lavage fluid from patients with sarcoidosis (mean 4.8:1) than from normal subjects (1.9:1) (fig 2b), though a wide range of values was seen. The proportion of T cells strongly expressing CD7 antigen (RFT2) and RF-DR was higher in patients with sarcoidosis than in control subjects though again a wide range of results was recorded (mean 21% (4%) v 7% (1%) for both; p < 0.05; fig 2b). There was a significant difference (p < 0.05) between lavage fluid from normal subjects and patients with sarcoidosis in terms of percentage of Tac+ T cells (9 (2) v 2 (0.4). Again, there was no difference in the percentages between smokers and non-smokers or between patient controls and normal volunteer controls.

RELATION BETWEEN IMMUNOCYTOLOGICAL
RESULTS AND SPECIFIC INDICES OF CLINICAL
DISEASE
There was no correlation between duration of disease, forced vital capacity, TLCO, or presence of a raised serum angiotensin converting enzyme concentration and the proportion of alveolar macrophages and lymphocytes expressing any of the markers studied (comparative data not shown).
Fig 2  (a) Proportion of total non-lymphoid mononuclear cells in lavage fluid from patients with sarcoidosis identified by specific monoclonal antibodies to macrophage subsets by means of the immunoperoxidase and double immunofluorescence methods used on cytospin preparations. Bars represent the range of results obtained from normal volunteers. Subjects in column 1 (RFD1/7) are also included in columns 2 and 3 (where RFD1 and RFD7 are expressed separately). (b) Proportion of total lymphoid cells in lavage fluid from patients with sarcoidosis expressing CD7 antigen, HLA-DR (RFDR), and CD25 antigen (anti-Tac). The relative proportions of CD4+ and CD8+ lymphocytes are given. Bars represent the range of results obtained from normal volunteers.

RELATION BETWEEN IMMUNOCYTOMETRY AND RADIOGRAPHIC STAGING OF SARCOIDOSIS

Patients with stage II and III chest radiographic shadowing had higher proportions of RFD1+ and RFD7+ macrophage like cells than those with a stage I chest radiograph (for both p < 0.05; fig 3) and a larger number of cells expressing both RFD1 and RFD7 (p < 0.005). No difference was found in RFDR+ macrophage like cells, but the proportion of RFD9+ and UCHM1+ cells was increased (p < 0.05 and p < 0.01 respectively; fig 3). When lymphocyte subsets were analysed no significant differences were found in CD4:CD8 ratios or RF-DR+ T cells. The lavage fluid of patients with a stage II or III chest radiograph had more RFT2+ (CD7) and Tac+ (CD25) T cells than the lavage fluid from patients with a stage I pattern (p < 0.05 and <0.01; data not shown).

IMMUNOCYTOLICAL FINDINGS RELATED TO DISEASE ACTIVITY

The proportion of RFDR+ macrophage like cells and RFD1+ cells in lavage fluid were similar in the patients.
judged to have active and inactive sarcoidosis (data not shown). Patients with active disease had higher percentages of RFD7* cells (51 (3-1) v 32 (7-6); p < 0.05), RFD9* cells (37 (5-6) v 13 (5); p < 0.05), UCHM1* cells (38 (5-2) v 9 (6-1); p < 0.005), and cells expressing both RFD1 and RFD7 antigens (41 (3-6) v 23 (1-9); p < 0.005) (fig 4) and higher percentages of CD7* and CD25* lymphocytes (p < 0.05 and < 0.01; fig 4).

Discussion

We have shown significant variations in the proportions of phenotypically distinct subsets of alveolar macrophages and lymphocytes between patients with sarcoidosis and normal subjects; the increase in the proportions of certain phenotypically distinct cells correlated with disease activity as determined by clinical criteria.

A very high proportion of lavage macrophages from patients with sarcoidosis coexpressed antigens recognised by the monoclonal antibodies RFD1 and RFD7. RFD1 recognises a cell membrane antigen related to HLA-DR, though not all HLA-DR cells coexpress RFD1 (for example, Langerhans cells). This suggests that RFD1 recognises an epitope with restricted expression. Chronic inflammatory diseases such as cryptogenic fibrosing alveolitis, rheumatoid arthritis, and eczema are characterised by the presence of increased proportions of RFD1* cells. The functional role of the RFD1 epitope in antigen presentation has been confirmed by studies where addition of the RFD1 monoclonal antibody inhibited the mixed lymphocyte reaction.

RFD7 antibody recognises a cytoplasmic antigen (77 kd) of mature tissue macrophages, which are usually also positive for acid phosphatase and which are efficiently phagocytic. The RFD7 antibody does not stain dendritic cells. Previous studies have shown that RFD1 and RFD7 antigens are separate in the developing fetus and are mutually exclusive in normal secondary lymphoid tissue, skin, and cultured peripheral blood monocytes. Coexpression of D1 and D7 has been previously found in diseases associated with chronic inflammation. We have now shown that a very small proportion of bronchoalveolar macrophages from normal subjects express both antigens; in patients with sarcoidosis this double expression is significantly increased. This result might indicate that such cells are immature and have not yet developed along separate pathways to express eventually one or the other antigen as mature cells. A second possibility is that dual antigen expression is the product of the local immune environment—that is, induced by lymphocyte interaction or soluble mediators. Classic RFD7* macrophages could, for example, be influenced by their environment to become RFD1*. Langerhans cells have been shown to
become RFD1\(^+\) when included in the lesions of atopic eczema.\(^{35}\) Whether this also results in functional change is not known. Certainly double expression of RFD1/D7 is substantially increased in sarcoidosis, and was associated with increasing disease activity and radiographic evidence of more extensive lung lesions. In most patients with sarcoidosis, especially those with active disease, the proportions of macrophages expressing RFD1, D7, and UCHM1 add up to more than 100%. Much of this “surfeit” of cells is explained by the double expression of RFD1 and RFD7. There is also, however, double (or more than double) expression of other macrophage markers. In most normal subjects the proportions add up to less than 100%, so that some of the macrophages must express none of these antigens. Furthermore, identification of these antigens depends on the expression of a sufficient concentration. The possibility remains that “negative” cells simply express small amounts of antigen below the detection threshold of the techniques used.

Increased numbers of activated T lymphocytes are also recovered in lavage fluid from patients with sarcoidosis, as evidenced by significantly higher proportions of strongly RFT2\(^+\) (CD7 cluster) lymphocytes than in lavage fluid from normal subjects. Some of these cells also express other activation markers—that is, interleukin-2 receptors (identified by the anti-Tac antibody against the CD25 antigen) and class II MHC (identified by the RFDR monoclonal antibody). Expression of all these antigens was increased on the alveolar lymphocytes from patients with sarcoidosis, especially those with active disease and stage II and III chest radiographs. Increased proportions of activated lymphocytes have been reported previously.\(^{1,2,11}\) Levels of lymphocytosis in lavage fluid have been suggested as being a good marker of disease activity,\(^{13,17}\) though other workers have cast doubt on this idea.\(^{19}\) The present study also fails to show correlations between lymphocytosis or lymphocyte activation and disease activity. Interestingly, relatively reduced levels of strong CD7 expression and HLA-DR expression occur in lavage fluid from patients with stage III disease. As this occurs at a time when the RFD1\(^+\)/RFD7\(^-\) macrophages form a major population in the lavage fluid, the possibility is suggested that this population in some way suppresses lymphocyte function. Such a possibility must, however, be tested by functional studies using isolated cells in vitro.

We have thus found higher proportion of alveolar macrophages expressing RFD1, RFD9, and UCHM1 and coexpressing RFD1/D7, and of alveolar lymphocytes expressing CD7 antigen and HLA-DR and interleukin-2 receptors in patients with sarcoidosis than in normal subjects. In patients with active disease and widespread lung lesions the increased proportions of RFD7\(^+\), RFD9\(^+\), UCHM1\(^+\), and RFD1\(^+\)/RFD7\(^-\) lavage fluid macrophages and of RFT2\(^+\) (CD7) and Tac\(^+\) (CD25) lavage fluid lymphocytes are particularly striking. None of the monoclonal antibody markers showed any correlation with disease duration, results of pulmonary function tests, or increased serum angiotensin converting enzyme activity when separate comparisons were made. Although these data suggest that lavage immunocytochemistry may provide clues to underlying immune pathogenetic mechanisms, and may be of value in assessing disease activity, results as yet do not prove or confirm that the assessment of surface phenotype can be used in determining prognosis or making therapeutic decisions for individual patients. Such conclusions await the results of controlled, prospective investigations of patients with this disease.

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References

 Relation between immunocytological features of bronchoalveolar fluid and clinical indices in sarcoidosis  


