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Bronchoalveolar lavage quality influences the T4/T8 ratio in sarcoidosis

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Summary
Background: Sarcoidosis is characterised by a T-lymphocytic alveolitis with a typically increased T4/T8 ratio. The diagnostic value of this ratio is under debate.
Aim of the work: We prospectively evaluated the influence of BAL pre-lavage and the impact of bronchial contamination on BAL differential cell count in 108 BAL specimens obtained from patients with histologically confirmed sarcoidosis.
Methods: BAL was performed by instilling 150–300 ml normal saline either in the middle lobe or the lingula. Fifty-one patients (47%) underwent additional pre-lavage with 50 ml normal saline. Bronchial contamination was assessed by semi-quantitative analysis of mucus, ciliated and squamous cells in the untreated BAL recovery.
Results: Pre-lavage did neither influence the lavage cellularity nor extend of contamination of the BAL. Content of mucus and ciliated cells, indicating bronchial contamination, showed a high correlation (Kendal's tau = 0.61). Presence of either mucus or ciliated cells in the BAL recovery was associated with a significant lower T4/T8 ratio (mucus: 4.9 vs. 8.0, $p = 0.009$; ciliated cells: 4.1 vs. 7.4, $p = 0.001$). Squamous cells in the BAL recovery representing oropharyngeal contamination did not significantly influence the T4/T8 ratio (7.7 vs. 5.6, $p = 0.10$).
Conclusion: Bronchial contamination of BAL as determined by the presence of mucus and ciliated cells in the recovery decreases the T4/T8 ratio of BAL in sarcoidosis.

Introduction

Sarcoidosis is a multisystemic granulomatous disease of unknown origin. The lung is involved in about 90% of patients

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of all age groups, presenting with a lymphocytic alveolitis, non-caseating granulomas, hilar adenopathy, pulmonary fibrosis, and pulmonary infiltrates.[1–4] Bronchoalveolar lavage (BAL) cytology in sarcoidosis shows a characteristic T-lymphocytic alveolitis with typically increased T4/T8 ratio.[2,5] The T4 lymphocytes are predominantly of TH1 phenotype producing interferon gamma and interleukin 2.[6] The higher the T4/T8 ratio the more it is specific for the diagnosis of sarcoidosis.[7,8] Its magnitude is influenced neither by the radiological stage of the disease, by patients race, gender, or age,[9] nor by the amount of the lavage volume.[10] Nevertheless, the T4/T8 ratio shows a high variability, and its diagnostic value for sarcoidosis is under debate.[8,9,11–14] In about 4–10% of cases, sarcoidosis present with a T8 lymphocytic alveolitis and a low T4/T8 ratio. T8 lymphocytes have been shown to make important contribution to the inflammatory process in pulmonary sarcoidosis.[15,16] Otherwise, the T4/T8 ratio has been regarded as prognostic factor in pulmonary sarcoidosis.[17,18]

BAL cell count displays a picture of the momentary intra-alveolar state, limiting the conclusions drawn from a single lavage result.[16,18,19] Non-resident alveolar cells including lymphocytes are in a permanent kinetic state with alveolar in- and efflux, proliferation, and apoptosis.[19,20] In sarcoidosis, alveolar cells show additional dynamics. Alveolar lymphocyte accumulation in sarcoidosis might be due to an enhanced mobility of lymphocytes, possibly mediated by alveolar macrophages.[21] While BAL cellularity shows interlobar variation in interstitial lung diseases, the BAL cell count is uniform in sarcoidosis throughout the lung.[22]

Besides technical processing of the lavage specimen, analysis of the cellular content of BAL is influenced by the quality of the obtained specimen.[23–25] The degree of extraalveolar contamination of the BAL can be assessed by the presence of broncho-epithelial material, e.g. squamous cells, ciliated cells, or mucopurulent exudates.[22,25]

Several techniques were introduced to improve the quality and reliability of BAL results, e.g. separating the first portion of the lavage to reduce the contamination of BAL specimen, or filtration of BAL recovery prior to cellular analysis.[23] However, none of these techniques are generally established in the clinical routine.

The present work prospectively evaluates the influence of pre-lavage and the impact of BAL contamination on differential cell count and lymphocyte subsets of the BAL in patients with histologically proven sarcoidosis.

Patients and methods

A total of 108 bronchoscopies with BAL were performed in 108 consecutive patients (56 male, 52 female) with histologically proven sarcoidosis according to the guidelines of the European Respiratory Society.[23]

Prior to the bronchoscopy, all patients received premedication with intravenous atropine, hydrocodeine, and midazolam, and inhalation of 4 ml of 4% lignocaine solution. Furthermore, 2% lignocaine was sprayed in the nasopharynx and oropharynx (3–4 times each). During the procedure, portions of 3 ml of 1% lignocaine were instilled over the vocal cords, in the trachea, and in both main bronchi, respectively. Another 3 ml of 1% lignocaine was instilled in the bronchus at the lavage site.

The bronchoscope was introduced transnasally or transorally. After inspection of the tracheobronchial tree, the tip of the bronchoscope was wedged in a subsegmental bronchus either in the middle lobe or the lingula. For performing BAL, the patient was brought in recline position, and 150–300 ml normal saline (NS) was instilled. Recovery was obtained by gravity when using a BAL volume of 150–300 ml or by gentle suction in BAL with a volume between 150 and 240 ml. The recovered fluid was collected in ice cooled silicone tubes for further preparation. The amount of the instilled lavage fluid and the recovered fluid was recorded.

Patients were alternately assigned for a pre-lavage with 50 ml NS prior to BAL in the same subsegmental bronchus. Pre-lavage was performed by instillation of 50 ml NS recovered by gentle suction. The recovered fluid of the pre-lavage has not been used for further cytological analysis.

Preparation of BAL recovery for analysis was performed using a standardised protocol. Briefly, the whole volume was centifuged twice, and a differential cell count was performed using a Neubauer-counting chamber. The following parameters were used in the cellular analysis: total cell count, percentage of macrophages, neutrophils, and lymphocytes. The sediment was divided, and one half was used for two Papanicolaou- and two Giemsa-stained smears, and one smear was stained for iron. The other half of the sediment was diluted in 10% fetal calf serum (FCS) with addition of 1 ml of mucoliquifying agent (Mucolexx). Cytospin specimens were prepared, air dried for 2 h at room temperature and then fixed in acetone for 10 min. The cytospin specimens were sealed and stored in the freezer at $-70\,^{\circ}C$ prior to immunocytochemistry. The alcaline phosphate (APAAP) technique was used for immunocytochemistry according to a standarised protocol. Anti-human CD4 antibody was applied to detect T4 cells (DAKO, Clone MT319, diluted at 1:20), and anti-human CD8 to detect T8 cells (DAKO, Clone DK25, diluted at 1:40) on the same slide as double immunostaining. Quantification of CD4 and CD8 positive cells was achieved by counting 2×100 cells each. The T4/T8 ratio was calculated by dividing the number of CD4 and CD8 positive cells.

Contamination of the BAL was assessed using three criteria according to the literature.[23,25] Content of mucus, ciliated cells, and squamous cells were evaluated semiquantitatively by light microscopy. Results were divided into four categories: 0 no, (+) little, + moderate, and ++ high contamination. The category "little" was defined, when mucus, ciliated cells, and squamous cells, respectively, were present in less than 1/3 of the microscopic area, the category "moderate" was used in cases with contamination detectable in not more than 2/3 of the microscopic area, and a "high"classification was considered in situation with contamination detectable in more than 2/3 of the microscopic area.

Category "0" and "(+)" were classified as no contamination, whereas "+" and "++" were defined as present contamination.

BAL differential cell count as well as degree of contamination was assessed by two independent investigators (B.K.

and B.B.). In dissimilar results, a final decision was made upon a 3rd assessment (P.D. or L.B.).

Statistics

Computerised statistical analysis was performed using SPSS version 8.0. and jmp statistics version 3.0. As the data were normally distributed, McNemar's test for correlation of paired data, Pearson correlation coefficient, Students *t*-test, and Chi-square test were used. For multivariate analyses, ANOVA were used including correction according to Bonferroni. Data are shown as mean (SD).

Results

A mean of 243 (64) ml NS was instilled. The mean fluid recovery was 137 (61) ml (55 (17)%, respectively). Cellular analysis showed a total cell count of 233 (184) $\times 10^6\,l^{-1}$ with a percentage of 61 (21)% macrophages, 35 (21)% lymphocytes, 3 (4)% neutrophils, and 0.6 (1)% eosinophils. Analysis of lymphocyte subsets showed 68 (18)% CD4 positive (T4) and 19 (15)% CD8 positive (T8) cells, corresponding to a T4/T8 ratio of 6.5 (6.0) (Table 1). There was no gender associated difference in differential cell count and count of lymphocyte subsets.

In 51 patients (47%), a pre-lavage was performed, whereas 57 patients did not receive a pre-lavage. This inhomogeneous distribution is caused by six patients assigned to the pre-lavage group, in whom the diagnosis of sarcoidosis has not been confirmed.

Performance of a pre-lavage did neither influence the differential cell count nor the contamination of BAL (Table 1).

The BAL recovery was similar in patients undergoing a BAL with 250–300 ml ($59 \pm 13\%$) compared to patient, in whom a BAL with 150–250 ml was performed ($55 \pm 11\%$) ($p = 0.06$). There were no correlations between instilled lavage volume and total cell count ($r = -0.14$), lymphocyte count ($r = -0.04$) or CD4/CD ratio ($r = 0.14$), respectively.

Assessing different contamination parameters, mucus and ciliated cells showed a high correlation (Kendal's tau = 0.61), whereas squamous cells did not correlate to ciliated cells and mucus material, respectively (Kendal's tau = 0.26 each).

We analysed the cellularity and the degree of contamination in respect to the BAL recovery in absolute (volume of returned fluid) and relative (% from the instilled lavage volume) values. There were no correlations between recovery and total cell count (absolute $r = -0.14$; relative $r = -0.12$), lymphocyte count (absolute $r = 0.07$; relative $r = 0.131$), or CD4/CD ratio (absolute $r = -0.1$; relative $r = 0.04$), respectively.

However, absolute and relative BAL recovery volume was significantly lower in samples with a high degree of contamination with mucus (ANOVA, $p < 0.005$), bronchial cells (ANOVA, $p < 0.01$), or squamous cells (ANOVA, $p = 0.01$). The cellular content of BAL was analysed in respect to the distinct contamination parameters. In the presence of squamous cells, the neutrophil count was slightly but significantly increased ($p = 0.04$), in contrast to other cell types (Table 2). When mucus was present in the BAL, the T4 cell count and the T4/T8 ratio were significantly decreased ($p < 0.03$ and $p = 0.005$, respectively; Table 3). BAL recovery contamination with ciliated cells was significantly associated with a decreased T4/T8 ratio ($p = 0.002$) due to a lower T4 cell count ($p = 0.05$), and a slightly increased neutrophil count ($p = 0.02$; Table 4).

The influence of mucus or ciliated cell contamination on T4/T8 ratio was not influenced by the performance of pre-lavage.

Discussion

BAL cellularity has an important impact on physicians diagnostic judgement in assessing interstitial lung disease.[26] The presence of BAL lymphocytosis and the analysis of lymphocyte subsets are of particular interest in sarcoidosis.[27,28]

The present study shows the influence of BAL contamination on the BAL cellularity in sarcoidosis. Bronchial contamination is best represented by the presence of mucus and ciliated cells, respectively, showing a strong

Table 1 Pre-lavage does not influence BAL quality and differential cell counts in sarcoidosis (mean (SD)).

	Total ($n = 108$)	With pre-lavage ($n = 51$)	No pre-lavage ($n = 57$)	
Total cell count ($10^6\,l^{-1}$)	233 (184)	236 (203)	229 (166)	0.86
Macrophages (%)	61 (21)	63 (19)	60 (23)	0.37
Neutrophils (%)	3 (4)	4 (5)	3 (3)	0.37
Lymphocytes (%)	35 (21)	32 (19)	37 (22)	0.22
T4+ (%)	68 (18)	66 (18)	71 (18)	0.20
T8+ (%)	19 (15)	18 (13)	19 (16)	0.63
T4/T8 ratio	6.5 (6.0)	6.3 (5.9)	6.6 (6.0)	0.79
BAL recovery (%)	55 (16)	57 (17)	53 (16)	0.22
Mucus (yes/no)	54/54	28/23	26/31	n.s.*
Squamous cells (yes/no)	66/42	31/20	35/22	n.s.*
Ciliated cells (yes/no)	36/72	14/37	20/37	n.s.*

n.s.: not significant.
*Chi-square test.

Table 2 Influence of squamous cells on differential cell counts in BAL (mean (SD)).

	No squamous cells ($n = 42$)	Squamous cells ($n = 66$)	p
Total cell count ($10^6\,l^{-1}$)	205 (112)	251 (217)	0.15
Macrophages (%)	65 (20)	59 (21)	0.16
Neutrophils (%)	2 (3)	4 (4)	0.03
Lymphocytes (%)	33 (20)	36 (21)	0.39
T4+ (%)	70 (18)	67 (19)	0.31
T8+ (%)	16 (11)	21 (17)	0.12
T4/T8 ratio	7.7 (6.7)	5.6 (5.2)	0.10
BAL recovery (%)	60 (16)	52 (16)	0.01
Ciliated cells (yes/no)	6/36	29/37	<0.01*
Mucus (yes/no)	16/26	38/28	n.s.

*Chi-square test.

Table 3 Influence of mucus on BAL differential cell counts (mean (SD)).

	No mucus ($n = 54$)	Mucus positive ($n = 54$)	p
Total cell count ($10^6\,l^{-1}$)	243 (202)	222 (165)	0.57
Macrophages (%)	60 (22)	62 (20)	0.57
Neutrophils (%)	3 (3)	4 (5)	0.03
Lymphocytes (%)	37 (22)	33 (20)	0.30
T4+ (%)	72 (16)	64 (20)	0.03
T8+ (%)	17 (13)	21 (16)	0.18
T4/ T8 ratio	8.0 (7.2)	4.9 (3.8)	0.009
BAL recovery (%)	58 (16)	52 (16)	0.06
Ciliated cells (yes/no)	4/50	31/23	<0.001*

*Chi-square test.

Table 4 Influence of ciliated cells on BAL differential cell counts (mean (SD)).

	No ciliated cells ($n = 73$)	With ciliated cells ($n = 35$)	p
Total cell count ($10^6\,l^{-1}$)	233 (186)	232 (182)	0.99
Macrophages (%)	60 (21)	64 (20)	0.33
Neutrophils (%)	3 (3)	5 (5)	0.03
Lymphocytes (%)	37 (21)	31 (20)	0.14
T4+ (%)	70 (18)	63 (19)	0.08
T8+ (%)	17 (15)	22 (14)	0.11
T4/T8 ratio	7.4 (6.5)	4.1 (3.3)	0.001
BAL recovery (%)	59 (15)	56 (16)	0.19

correlation. Squamous cells in the BAL recovery might be merely due to upper airway contamination.[4]

Separation of the first aliquot of the lavage as ''pre-lavage'' with 50 ml NS did neither influence the amount of bronchial contamination nor the cellular composition of recovered BAL. Therefore, we could not confirm the usefulness of the separation of the first lavage aliquot as recommended by others,[24] and based on our experience we stopped to perform pre-lavage in our institution.

Bronchial contamination of BAL did significantly influence the T4/T8 ratio. We found a significantly lower T4/T8 ratio in BAL specimen with a high degree of bronchial contamination as displayed by content of mucus and ciliated cells. Therefore, we regard the assessment of contamination important for evaluation of BAL results. As filtration of the BAL recovery removes contamination, it should be considered not to filter the whole lavage specimen in sarcoidosis. This has been previously proposed in bronchial asthma.[29]

BAL cellular analysis is dependent on the quality of the obtained lavage specimen. In our study, the degree of contamination was higher when only a small BAL recovery could be retrieved. This supports previous findings that an appropriate lavage specimen should be recovered.[23,30]

BAL cellularity is influenced by the technical preparation of BAL recovery. When using cytocentrifugation, counting in a circular pattern around the centre area is required, as cells and especially lymphocytes might not equally distribute over the cytocentrifuged spot.[24,31] This factor can be eliminated by using a standardised protocol as in our study. The technique of cellular analysis itself, either immunostaining of FACS-based analysis seems not to influence the cellular analysis.[32,33]

Previous studies have proposed the value of induced sputum cytology in sarcoidosis; however, the results are varying between different investigators.[34,35] One study showed a lower lymphocyte count when induced sputum was contaminated by the bronchial and oropharyngeal compartment, indicating that the issue of contamination needs to be taken into account also when using different techniques for assessment of alveolar cellularity.[34]

About 4–10% of patients with pulmonary sarcoidosis present with a T8 lymphocytic alveolitis. It remains speculative, if some of our patients may have a low T4/T8 ratio due to a T8 alveolitis. However, the lower T4/T8 ratio was merely due to a lower T4 cell count rather than increased T8 lymphocytes. Furthermore, the incidence of 4–10% T8 lymphocytic alveolitis would potentially affect less than 10 BAL in our study.[11,15,16]

There are several limitations of our study that need to be addressed. Firstly, BAL was performed with a wide range of infusion fluid. The amount of fluid was individually determined during the investigation. Secondly, the BAL differential cell count has not been counter-checked by another method, e.g. FACS-analysis. However, we used a standardised protocol for cellular analysis and immunostaining, which might minimise the risk of error. Nevertheless, we included all patients with sarcoidosis assessed at our institution in the investigation to avoid selection bias.

In conclusion, in sarcoidosis the T4/T8 ratio of BAL is significantly influenced by bronchial contamination, represented by the presence of mucus and ciliated cells in the lavage recovery. Bronchial contamination needs to be considered when interpreting BAL results.

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