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Proliferating alveolar macrophages in BAL and lung function changes in interstitial lung disease

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ABSTRACT: In interstitial lung disease, the number of alveolar macrophages (AMs) can be increased. This may be caused by recruitment of precursor cells from peripheral blood and/or local proliferation in the lung.

We therefore analysed proliferation, by studying both the expression of the nuclear proliferation antigen, Ki67, and the deoxyribonucleic acid (DNA) content, using the Feulgen reaction followed by cytometry. The patients had interstitial lung disease, *i.e.* sarcoidosis (n=20), extrinsic allergic alveolitis (n=20), idiopathic lung fibrosis or lung involvement in collagen-vascular disease (n=19).

In all patient groups there was a significant increase in proliferating AMs compared to healthy controls (4.2 *versus* 1.4% Feulgen, 2.1 *versus* 0.5% Ki67), with a significant correlation between these two parameters. A positive correlation was also found in bronchoalveolar lavage (BAL) between numbers of lymphocytes and proliferating cells in sarcoidosis and in fibrosis. In fibrosis, numbers of eosinophils and proliferating cells were also positively correlated. Our main finding was, however, a positive correlation between numbers of proliferating cells (Feulgen) and lung function parameters, especially vital capacity and oxygen tension (Po_2) at rest, in patients with sarcoidosis and lung fibrosis. By contrast, in extrinsic allergic alveolitis, no correlation could be observed between proliferating cells and cell population or lung function.

Our results suggest that local proliferation of macrophages is an important element in interstitial lung disease.

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Alveolar macrophages (AMs) play an important role in the pathophysiology of interstitial lung diseases, since they are involved in cellular immunity, by interaction with lymphocytes and other cells [1]. Furthermore, they can release numerous mediators, which are important for initiation and maintenance of local inflammation [2, 3]. AMs belong to the monocyte system, and most AMs appear to be derived from blood monocytes, which migrate into the lung. In addition, AMs can proliferate locally when driven by growth factors, such as granulocyte macrophage colony stimulating factor (GM-CSF) [4, 5]. In alveolitis, the AMs appear to be activated, as evidenced by upregulation of several cell surface molecules. Since functional activation can go along with cell proliferation, as reported for *in vitro* experiments and an animal model [6–8], we looked, in this study, for AM proliferation under conditions of local inflammation in interstitial lung disease.

We report here, for the first time, that quantitative deoxyribonucleic acid (DNA) measurement by Feulgen reaction [9], followed by cytometry, can demonstrate local proliferation of alveolar macrophages in bronchoalveolar lavage (BAL). We compared these results with the immunocytochemical detection of Ki67 expression [10] in this population, and correlated our results with characteristics from BAL and clinical data.

Patients and material

Study population

Twenty patients with extrinsic allergic alveolitis (EAA) (9 with farmer's lung, 11 with bird fancier's lung, 19 nonsmokers, 1 smoker) were investigated. The diagnosis was based on a history of antigen exposure, typical clinical features of acute disease precipitating antibodies against the relevant allergens, and histological examination of lung biopsies. The group of sarcoidosis patients consisted of 20 patients (15 nonsmokers, 5 smokers), diagnosis was based on histological examination of lung biopsies.

Only sarcoidosis patients with lung function impairment were included in this study. The group of patients with lung fibrosis (nonsmokers) consisted of two subgroups: 14 patients with idiopathic lung fibrosis and 5 patients with lung involvement of collagen-vascular disease. Diagnosis was based on histological examination of lung biopsies and on the pattern of anti-antibodies. For further clinical details, see table 1. Control donors (nonsmokers) were apparently healthy volunteers (n=6). The study had the approval of the Ethics Committee of the University of Munich, Medical Faculty.

Methods

Bronchoalveolar lavage

After informed consent had been obtained, all patients underwent fibreoptic bronchoscopy and BAL. Lavage was performed by instilling 160 ml of 0.9% saline solution, in 20 ml aliquots, into the lingula or middle lobe, and by withdrawing the fluid immediately. Total cell counts were determined, and cytocentrifuge samples were prepared for cytological and immunocytochemical analysis. For determination of percentages of lymphocytes and macrophages, differential counts of 100 cells were made (Wright-Giemsa staining). The BAL characteristics of the different study populations are shown in table 2.

Immunocytochemistry

For immunocytochemical staining, monoclonal antibodies were used, in conjunction with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [11]. For identification of a nuclear proliferation antigen, the monoclonal antibody Ki67 [10] was used, with a CD8

Table 1. – Clinical data of patients' groups

Group	Age yrs	Sex M/F	S/NS	Treated with CS	X-ray changes	VC %*	Dco %*	Po_2 at rest⁺ mmHg
Sarcoidosis n=20	35±12	12/8	5/155		†Stage I n=6 Stage II n=8 Stage III n=3	85±18	80±23	-3.5±5.3
Fibrosing lung disease n=19	60±30	6/13	0/19	8	Diffuse opacities	73±18	68±27	-6.1±7.8
EAA n=20	49±29	10/10	1/19	1	Diffuse opacitites	82±16	66±20	-5.6±7.2
Controls n=6	32±3	3/3	0/6	-	ND	ND	ND	ND

Data for age, VC, Dco and Po_2 are presented as mean±SD. *: of predicted value; CS: corticosteroids; EAA: extrinsic allergic alveolitis; ND: not done; S: smoker; NS: nonsmoker; VC: vital capacity; Dco: diffusion capacity for carbon monoxide; Po_2: oxygen tension. †: X-ray staging. ⁺: difference from predicted value.

Table 2. – BAL differential cell count and ratio of T-cell subsets in patients with interstitial lung diseases and healthy controls

	Total cell count $\times 10^6$	Alveolar macrophages	Lymphocytes	Neutrophils	Eosinophils	CD4/CD8 ratio
Healthy controls n=6	4.8±0.6	90.0±0.8	8.0±1.9	2.0±0.5	0	ND
Sarcoidosis n=20	24.3±3.3	57.0±3.2	37.0±3.6	4.0±1.0	2.0±0.6	4.4±0.4
EAA n=20	35.0±6.1	41.0±3.8	44.0±5.4	12.0±3.9	3.0±0.6	0.50±0.6
Fibrosing lung diseases n=19	17.0±2.1	56.0±4.0	29.0±4.2	12.0±2.2	3.0±0.9	0.8±0.4
Idiopathic lung fibrosis n=14	17.5±2.4	56.0±4.3	31.0±5.1	10.0±3.0	3.0±1.0	0.7±0.1
Collagenous vascular disease n=5	15.5±5.4	56±10.5	26.0±7.8	15.0±2.9	3.0±2.3	0.8±0.3

Data are presented as mean±SD. EAA: extrinsic allergic alveolitis; ND: not done.

monoclonal antibody as an isotype control. T-cell subsets were identified with CD4 and CD8 monoclonal antibodies (MoAbs) (all MoAbs, Dakopatts, Hamburg, Germany). For the enumeration of Ki67 positive AM, 400 cells were counted by two independent investigators, and values are expressed as % of AM.

Feulgen reaction

The Feulgen reaction was carried out according to the Azure A staining protocol by Becton-Dickinson (Heidelberg, Germany). Firstly, the slides were fixed with paraformaldehyde. Hydrolysis was performed for 60 min with 5 N hydrochloric acid. Then, the slides were treated with Schiff's reagent (Azure A) for 1 h, and washed in SO_2 water three times for 10 min. After dehydration, the cells were embedded with Eukitt (O. Kindler, Freiburg, Germany).

Cytometry

DNA content was measured by a digital image analysis system, CAS 200 (Becton Dickinson). This was composed of a microscope (Reichert, Diastar), objective magnification ×40, a CCD-TV camera and an IBM AT computer, with the QDA program version 2.0, which performs a standardized measurement of DNA content of single cells in histology and cytology. For calibration of each measurement, slides with rat hepatocytes were treated in the same way as BAL cells. Thirty hepatocytes were measured for standardization. Additionally, as a reference, DNA content of chicken erythrocytes was measured. On each slide, as an internal standard, 30 lymphocytes were measured. Then 200 alveolar macrophages were segmented semi-automatically. The selected images were digitized in 6×2 pixels, resulting in pixel distances of 0.2 μm^2. Transmission was performed pixelwise into extinction, and digitized into 256 channels [12].

The following criteria were measured: objectivation of G_o/G_1 phase of alveolar macrophages, determination of 2c, 3c and 4c, and enumeration of cells undergoing S and G_2 phase. Cells in S, G_2 and M were considered to be proliferating cells and expressed as % of the AM counted.

Statistical analysis

For statistical analysis, the Pearson test, Student's t-test, and the nonparametric Wilcoxon test were used. Mean±SD values are given.

Results

Ki67 antigen expression in AMs

Typical nuclear staining for Ki67 was observed in control donors in only a minor percentage of AMs (0.5±0.6%). By contrast, in sarcoidosis the percentage was much higher (2.3±2.0%) (p=0.05) (fig. 1), and here patients with higher X-ray stages tended to have higher values. In patients with fibrosis, an average of 2.4±2.2% of the cells were positive for Ki67 (p=0.005), and in EAA patients, 1.8±1.8% (NS). There was no significant difference between smokers and nonsmokers concerning Ki67 expression, but there was a significant difference between patient groups and healthy controls (2.1 *versus* 0.5% Ki67, respectively p=0.05).

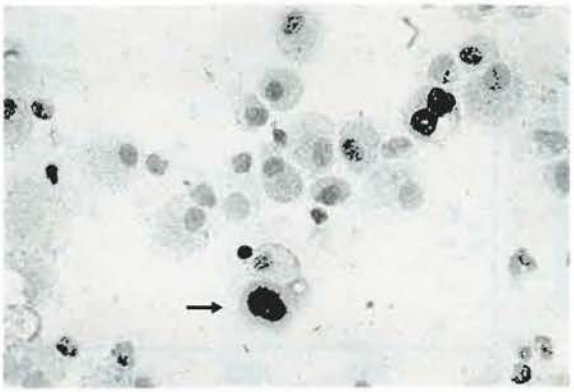

Fig. 1. – Expression of Ki67 antigen in alveolar macrophages in sarcoidosis. Cytospin preparations of lavage cells by alkaline phosphatase anti-alkaline phosphatase (APAAP) technology with a Ki67 monoclonal antibody. Arrow indicates a Ki67⁺ alveolar macrophage.

DNA content in AM

Using Feulgen stained samples, control donor's AMs had 1.4±0.5% proliferating cells. In sarcoidosis, the percentage was significantly higher (4.0±1.6%). In fibrosis, 4.3±1.6% proliferating cells were observed, and EAA patients also had an average of 4.3±1.5%. No significant differences concerning AM proliferation could be detected between smokers and nonsmokers, but there was a significant difference between healthy controls and all patient groups (p<0.00001).

Comparison of Ki67 staining and DNA analysis

Analysis of the combined data from all patients and controls showed a good correlation between Ki67 antigen expression and DNA analysis (r=0.6, p=0.0001).

Correlation between proliferating cells and cell populations in BAL

A positive correlation was found between percentage of proliferating cells (Feulgen) and lymphocyte numbers, in both sarcoidosis and fibrosis, but not in EAA (fig. 2 and table 3). In fibrosis only, there was, in addition, a positive correlation between proliferating cells (Feulgen) and numbers of eosinophils (fig. 2 and table 3). Although Feulgen staining correlated with Ki67 staining, Ki67 expression did not correlate with any of the parameters.

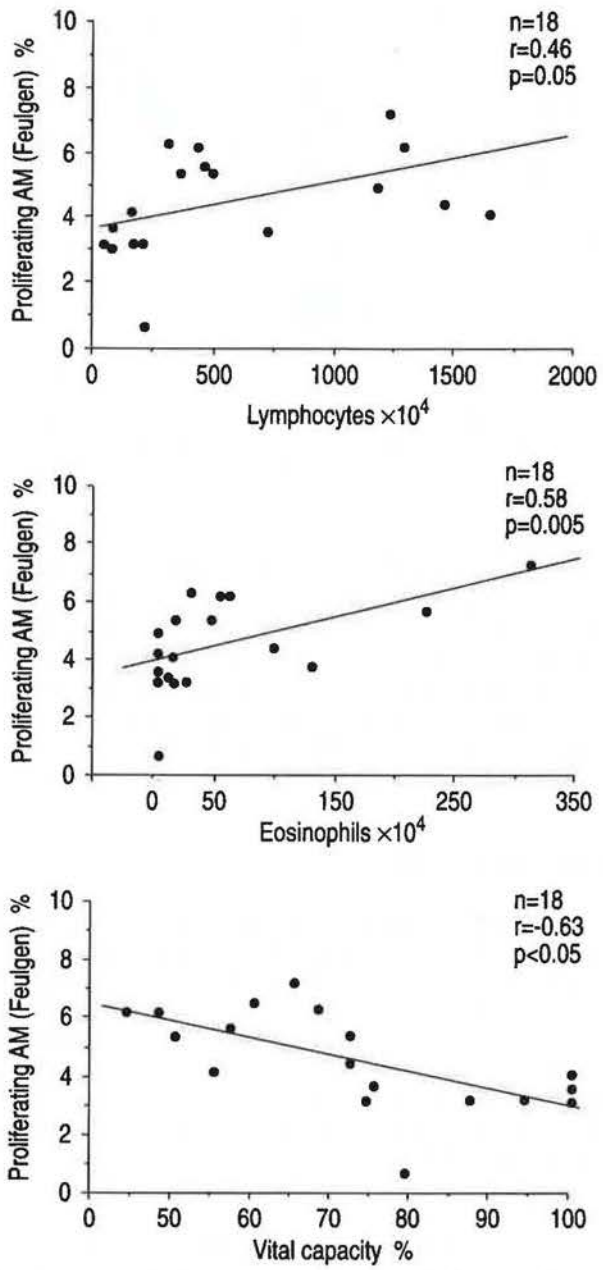

Fig. 2. – Correlation between proliferating alveolar macrophages, lymphocytes and eosinophils in bronchoalveolar lavage (BAL) fluid, and reduction of vital capacity (% pred) in lung fibrosis (total cell count available in 18 out of 19 patients).

Correlation between proliferating cells and lung function tests (table 3)

In sarcoidosis, there was only a loose positive correlation between proliferating cells and vital capacity (VC) and diffusing capacity for carbon monoxide (Dco) (p=0.07 and 0.06 NS, respectively), whilst a significant negative correlation was found between proliferating cells and oxygen tension at rest (Po_2) at rest. In fibrosing lung disease, there was an inverse relationship between proliferation index (% proliferating cells) and VC, Dco, and Po_2 at rest. In EAA, there was no correlation between proliferation index and lung function parameters.

Discussion

Although AMs are differentiated tissue macrophages, they can proliferate under certain conditions [4]. In this study, we could demonstrate a significantly increased rate of proliferating AMs in BAL of patients with interstitial lung disease, using both Feulgen and Ki67 staining. A previous report on measurement of DNA synthesis in patients with interstitial lung diseases revealed higher levels in sarcoidosis compared to fibrosis patients [5], while no data on AM proliferation in EAA are available in the literature. Increased percentage of $Ki67^+$ cells have previously been observed in sarcoidosis patients [13, 14]. In animal models, macrophage proliferation could be demonstrated after stimulation of lavage cells [6, 15].

In interstitial lung diseases, functional activation of AMs can be observed. A characteristic finding is the pronounced expression of cell surface receptors, such as the transferrin receptor and interleukin-2 (IL-2) receptor [16, 17], and an increased production of cytokines, such as tumour necrosis factor (TNF) and interleukin-1 (IL-1) [18, 19]. These cytokines, in turn may induce other growth factors, such as macrophage colony stimulating factor (M-CSF) and GM-CSF [20], which can be found in BAL of patients with sarcoidosis [13], and allergic asthma [21]. The role of GM-CSF in macrophage proliferation has been intensively investigated, especially in the animal model. In mice, transgenic for GM-CSF, an excessive increase in peritoneal macrophages could be demonstrated, with a high mitotic rate [7]. The stimulatory effect of lymphocyte products, such as IL-2 and GM-CSF, on AM proliferation might be important in the pathophysiology of the disease, since we found a close

Table 3. – Correlation between numbers of proliferating alveolar macrophages (Feulgen) and 1) lung function parameters and 2) BAL charateristics in patients groups

		VC	Dco	Po_2 at rest	Lymphocytes	Eosinophils
Sarcoidosis	p=	NS	NS	0.005	0.0001	NS
n=20	r=	0.49	-0.57	-0.72	0.714	-0.24
Fibrosing lung	p=	0.005	NS	0.05	0.03	0.005
Disease n=19	r=	-0.63	-0.52	-0.47	0.46	0.58
EAA	p=	NS	NS	NS	NS	NS
n=20	r=	-0.22	-0.29	+2.00	-0.02	-0.27

NS= not significant; BAL: bronchoalveolar lavage. For further abbreviations see table 1.

correlation between numbers of lymphocytes and AM proliferation index in sarcoidosis and fibrosing lung disease. Another interesting finding was the positive correlation between numbers of eosinophils and AM proliferation index in fibrosing lung disease. The numbers of eosinophils in BAL have been shown to be of predictive value for the clinical disease outcome in fibrosing lung disease [22].

The major finding in this study was the positive correlation between AM proliferation index and lung function impairment in sarcoidosis and fibrosing lung disease. Since, in sarcoidosis a pronounced influx of small monocyte-like CD14$^+$ cells has been reported [23], we looked for a correlation between size and signs of proliferation in AMs, but, interestingly, not only small AMs with a high nucleus-cytoplasm-relation, but also large differentiated AMs (fig. 1), were positive for Ki67 expression and proliferation.

EAA patients represent an exception in this study, since no correlation between BAL lymphocytosis and AM proliferation index could be detected. It must be taken into account that the characteristic finding in BAL of EAA patients with high lymphocyte numbers is not correlated to clinical symptoms anyway, since these abnormalities will also be found in healthy exposed persons [24]. Also, no correlation could be found between lung function tests and AM proliferation, probably for the same reason.

Despite the close correlation between Ki67 antigen expression of AMs and proliferation index obtained by Feulgen staining, values for DNA staining were higher than those for Ki67 antigen expression. This agrees with results found in cultured human bone marrow stem cells, which exhibited high levels of DNA synthesis, but low Ki67 antigen expression [25].

Measurement of proliferation index may give additional information about lung involvement in sarcoidosis and fibrosing lung disease, since our data suggest that local proliferation of macrophages is an important element in interstitial lung disease.

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