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Correlation of morphological patterns of nucleoli in alveolar macrophages with HLA-DR antigen expression in sarcoidosis

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Abstract

Background **Alveolar macrophages from patients with sarcoidosis express increased quantities of HLA-DR during activation. Because silver staining has been described as a sensitive indicator of cellular activity a study was performed to examine whether it relates to HLA-DR antigen expression.**

Methods **The relation between silver staining patterns of nucleoli and HLA-DR antigen expression was examined in alveolar macrophages collected by bronchoalveolar lavage from 11 patients with pulmonary sarcoidosis and 11 control subjects.**

Results **The mean (SD) number of silver stained protein dots associated with the nucleolar organiser regions (AgNORs) was significantly higher in alveolar macrophages from patients with sarcoidosis (7·5 (1·5)) than in those from control subjects (5·6 (0·6)). The number of silver stained dots in alveolar macrophages correlated significantly with the intensity and the density of HLA-DR antigen expression in the patients with sarcoidosis.**

Conclusion **Silver staining may be a sensitive tool for the investigation of the biological cell activity of alveolar macrophages in sarcoidosis.**

Silver staining of nucleoli was first reported in 1899.[1] A local and functional relation was found between certain chromosomes and nucleoli;[2] the regions thus identified have been called nucleolar organiser regions (NORs).[3 4] These are regions of acrocentric chromosomes containing major ribosomal RNA (rRNA) genes and NOR associated proteins, which result from transcription[5–8] and can be seen with silver staining. Previous studies have related the amount and distribution of NOR associated silver stained proteins with cellular activity in vitro[9–13] and in vivo.[14–16] The silver stained dots are usually called AgNORs, and should not be confused with the number of transcriptionally active NORs in the cell. Wide discrepancies have been observed between the number of silver stained dots and the number of NOR chromosome genomes.[10 17 18] In many studies silver staining has been found to be helpful in the diagnosis of malignancy and as a sensitive indicator of cellular activity.[6 19–21]

Alveolar macrophages in patients with sarcoidosis are described as being highly activated and are concerned in an active immunological process.[22–26] They have been shown to express increased quantities of HLA-DR antigen during activation. The fact that silver staining is also a sensitive marker of cellular activation prompts the question of whether silver staining is correlated with HLA-DR expression, an immunological feature of activated alveolar macrophages. The object of our investigation was to compare silver staining and HLA-DR expression in alveolar macrophages obtained by bronchoalveolar lavage from patients with sarcoidosis and from healthy control subjects.

Methods

SUBJECTS

We studied 11 patients with pulmonary sarcoidosis (confirmed by transbronchial lung biopsy) and 11 healthy control subjects. Consecutive patients with sarcoidosis seen in the pulmonary unit were included provided that they had not been treated and were non-smokers at the time of the investigation; two patients were ex-smokers who had smoked less than 10 cigarettes a day and stopped at least two years before the investigation. The control subjects were healthy non-smokers, three of whom had smoked less than 10 cigarettes a day but had stopped at least three years before the investigation. The diagnosis of sarcoidosis was confirmed by transbronchial lung biopsy.

BRONCHOALVEOLAR LAVAGE

All subjects underwent bronchoalveolar lavage,[27] which was performed with a fibreoptic bronchoscope (Olympus B10) after local anaesthesia with 2% xylocaine. One hundred millilitres of isotonic warmed saline were instilled into the middle lobe in 20 ml aliquots and recovered by gentle aspiration. The fluid was collected into siliconised glass tubes and processed immediately at 4°C.

HLA-DR ANALYSIS

Cells were counted in a haemocytometer. Cytocentrifuge cell preparations were made for May-Grünwald-Giemsa and silver staining. Differential cell counts were performed on 400 cells and individual counts were given as per-

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*Table 1 Data on the subjects and results of analysis of bronchoalveolar cells (mean (SD) values)**

	Sarcoidosis (n = 11)	Controls (n = 11)	p
Age (years)	47·5 (8·2)	45·6 (9·9)	
Sex (M/F)	2/9	2/9	
Chest radiograph stage†			
I	3	0	
II	6	0	
III	2	0	
Respiratory values (% pred)			
VC	87·9 (19·9)	97·6 (11·7)	NS
FEV_1	82·9 (17·0)	95·5 (13·4)	NS
TLC	100·5 (15·1)	110·8 (8·3)	<0·05
TLCO	78·2 (12·1)	96·2 (13·5)	<0·05
TLCO/VA	73·4 (11·1)	96·3 (14·5)	<0·01
ACE (U/ml)	23·8 (8·6)	13·0 (4·3)	<0·05
Lavage fluid total cell count ($\times 10^6$)	16·9 (9·4)	10·1 (2·5)	<0·05
Differential count (%)			
Alveolar macrophages	52·6 (23·8)	88·9 (7·2)	<0·05
Lymphocytes	45·0 (25·7)	8·6 (7·2)	<0·05
Neutrophil granulocytes	2·0 (2·6)	1·0 (1·0)	NS
Eosinophil granulocytes	0·4 (0·4)	0·4 (0·7)	NS
Alveolar macrophages			
% HLA-DR positive	97·8 (1·2)	96·5 (2·5)	NS
Cell diameter (μm)	24·5 (1·6)	23·3 (1·3)	NS
Nuclear diameter (μm)	9·0 (0·6)	9·1 (0·4)	NS
No of IF units	183·6 (42·0)	122·0 (29·9)	<0·05
Density of IF units	0·0972 (0·0191)	0·0730 (0·0212)	<0·05
No of silver positive dots/nucleus	7·5 (1·5)	5·6 (0·6)	<0·01

*Differences between the sarcoidosis group and the control group analysed by the Kolmogoroff-Smirnoff test.
†Sarcoidosis stage: I—bilateral hilar lymphadenopathy; II—I + infiltrates; III—lung fibrosis.
VC—vital capacity; FEV_1—forced expiratory volume in one second; TLC—total lung capacity; TLCO—transfer factor for carbon monoxide; TLCO/VA—transfer factor for carbon monoxide per alveolar volume; ACE—serum angiotensin converting enzyme; IF—immunofluorescence units.

centages of the cell total. Alveolar macrophages were identified by their light scattering properties in a cytofluorometer (Ortho Cytoron); autofluorescence was measured by means of a red fluorescence high pass filter (630 nm). HLA-DR antigen expression was examined after immunofluorescent staining by subjecting at least 1000 alveolar macrophages to cytofluorometry. For this procedure aliquots of 0·5 ml of lavage fluid were incubated with 25 μl of OKDR-phykoerythrin conjugate (Ortho Diagnostic). The number of HLA-DR antigen positive alveolar macrophages was expressed as a percentage of the total number of alveolar macrophages. The intensity of immunofluorescent staining was referred to as immunofluorescence (IF) "units." Total cell size and nuclear size were measured, and the density of immunofluorescence was calculated from the results of immunofluorescent staining and cell size.[25]

SILVER STAINING AND NUCLEOLAR MORPHOLOGY

After the slides had been fixed in phosphate buffered formalin silver staining was done with freshly prepared reagent: 2 g gelatin dissolved in a 1% aqueous solution of formic acid was mixed with a 50% aqueous solution of silver nitrate in a proportion of 1:2.[20] The cytocentrifuge cell preparations were incubated in this reagent for 30 minutes at room temperature under dark room conditions. The slides were then washed thoroughly with deionised water, dehydrated in xylene, and mounted in Entelan. The number of silver stained dots (AgNORs) per nucleus was determined for each subject from 400 alveolar macrophages without knowledge of the diagnosis.

ANALYSIS

The Kolmogoroff-Smirnoff test was used to compare the two groups and Spearman's rank correlation to study the influence of cellular changes and HLA-DR expression on the AgNOR counts. A p value below 0·05 was considered significant.[28]

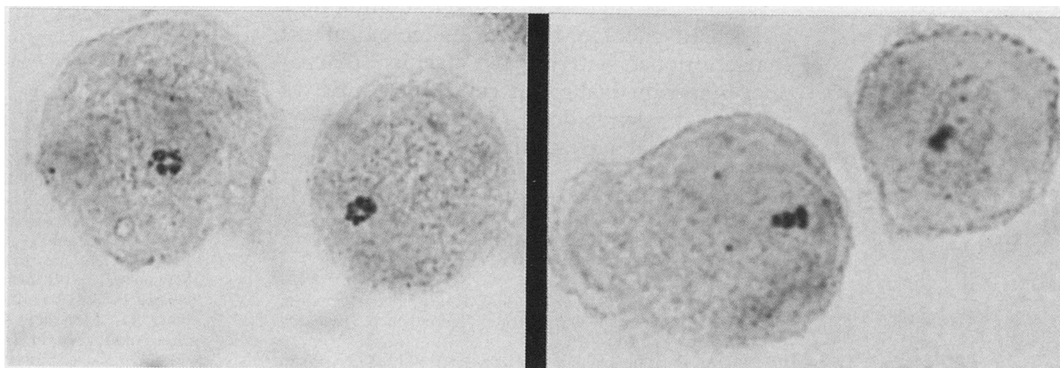
Results

Details of the subjects and the results are shown in table 1. Patients with pulmonary sarcoidosis had higher total cell counts, lower percentages of alveolar macrophages, and higher percentages of lymphocytes. The proportion of HLA-DR positive alveolar macrophages was above 90% in both groups. The intensity of immunofluorescent staining of HLA-DR antigen varied considerably but was significantly higher in the patients with sarcoidosis. The number of AgNORs within the nuclei of alveolar macrophages was significantly higher in the subjects with sarcoidosis than in the control subjects (figure).

There was a significant positive correlation between the number of AgNORs and both the intensity of immunofluorescent staining of HLA-DR antigen and the density of HLA-DR antigen expression on alveolar macrophages in the patients with sarcoidosis (table 2). The trend was in the same direction in control subjects but was not significant. The number of AgNORs did not correlate with the percentages of DR positive alveolar macrophages, total cell size, or nuclear size.

Discussion

Silver staining of alveolar macrophages



Left: Alveolar macrophages collected from a patient with sarcoidosis obtained by bronchoalveolar lavage showing several silver stained dots within the nucleolus. Right: Alveolar macrophages from a control subject showing only a few silver stained dots within the nucleolus.

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Table 2 *Spearman's rank correlation analysis**

	Silver stained dots (AgNORs)/nucleus	
	r	p†
Sarcoidosis group		
Mean % of HLA-DR positive cells	0·00235	NS
Mean cell diameter	0·47273	NS
Mean nuclear diameter	0·24146	NS
Mean IF units	0·87016	<0·001
Mean density of IF units	0·64546	<0·05
Control group		
Mean % of HLA-DR positive cells	−0·01839	NS
Mean cell diameter	−0·11818	NS
Mean nuclear diameter	0·51618	NS
Mean IF units	0·41914	NS
Mean density of IF units	0·35455	NS

*Correlations of silver stained dots with HLA-DR antigen expression on alveolar macrophages are given for patients with sarcoidosis and controls.
†Spearman's rank test.
IF—immunofluorescence.

obtained from patients with pulmonary sarcoidosis showed an increased number of dots. These silver stained dots or AgNORs[15 16 19–21] represent proteins associated with the nucleolar organiser regions.[29 30] The relatively large number of AgNORs in alveolar macrophages seems to be a typical feature of these cells. In previous studies the location of the silver stained dots has coincided with the fibrillar centres and the dense fibrillar components of the nucleoli. AgNORs have been counted in different conditions and, although a strictly numerical correlation between rDNA transcription of nucleolar organiser regions and the number of AgNORs is not present, silver staining is considered to be a sensitive marker of cell proliferation and metabolic activity.[9 18 30]

In the patients with sarcoidosis the number of AgNORs per nucleus was found to correlate with higher intensity and density of HLA-DR expression of alveolar macrophages. No relation was found between the number of AgNORs and the percentage of HLA-DR positive alveolar macrophages, perhaps because of the high percentage of positive cells in both patients and control subjects. Moreover, no correlation was found between the size of cell or nucleus and the AgNOR counts. HLA-DR antigen is expressed in antigen presenting cells, reflecting increased cellular activity and being related to the primary immune response.[22 23 25 31–33] The positive correlation between the number of AgNORs per cell nucleus and HLA-DR antigen expression in alveolar macrophages in sarcoidosis probably indicates increased metabolic cell activity.

Our results suggest that silver staining may be a sensitive tool for monitoring biological and transcriptional activity of alveolar macrophages in non-malignant conditions, such as pulmonary sarcoidosis, characterised by immunological activation.

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