The phenotype of alveolar macrophages and its correlation with immune cells in bronchoalveolar lavage


ABSTRACT: The phenotype of alveolar macrophages (AMs) is known to be modulated during pathological immune reactions in the lung. In this study, we wanted to determine the relationship between the AM phenotype and changes in the proportions of the various immune cells in bronchoalveolar lavage (BAL).

BAL was performed in 76 consecutive patients, including 32 with sarcoidosis, 8 with idiopathic pulmonary fibrosis, 9 with pneumoconiosis, 13 with other respiratory disorders, and 14 controls without evidence of interstitial lung disease. The phenotype of AMs was studied by a panel of 15 monoclonal antibodies against various myeloid antigens, and was correlated with the proportions of cells obtained by BAL.

The percentage of BAL lymphocytes showed a relationship with the expression of macrophage antigens in 11 out of 15 antigens studied (all except adhesive molecules CD11a, CD11c, CD18 and the antigen 2SF9 present on mature macrophages). Furthermore, the CD4/CD8 ratio of BAL T-lymphocytes correlated with the AM expression of CD54 (intercellular adhesion molecule-1 [ICAM-1]), RFD1 (marker of dendritic cells), and CD71 (transferrin receptor). In samples with an increased number of bronchoalveolar neutrophils, the subpopulation of 27E10 positive AMs (inflammatory acute phase macrophages) was increased. Eosinophils in BAL were not associated with a significant increase in AM membrane antigen expression. Prominent changes of the AM phenotype were found in active sarcoidosis showing lymphoctic alveolitis, with more frequent expression of CD54, KiM2, CD71, CD11b and RFD9.

In conclusion, this study shows that the phenotype of AMs is related to the type and intensity of the immunopathological reaction in the lung, and correlates with the proportions of bronchoalveolar cells.

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Alveolar macrophages (AMs) represent a heterogeneous population of mononuclear phagocytes, reflecting different stages of activation and differentiation [1-3]. These cells are able to change their phenotypic pattern [4] and functional properties [5] in response to exogenous stimuli. Modulation of membrane antigens probably helps AMs to effectively regulate their scavenger activity in the lung microenvironment.

Membrane glycoproteins of the integrin family, which are currently being extensively studied on AMs [6-8], are of fundamental importance for the macrophage migration, anchorage, and normal effector functions [9]. In particular, β₂-integrins (CD11a/CD18, leukocyte integrins) and their ligands seem to be involved in cell-cell interactions of bronchoalveolar cells, and their membrane expression can vary with respect to the pathological processes in the lung parenchyma [10, 11]. In contrast to many adhesive molecules which are expressed on both monocytes (the precursors of AMs in the peripheral blood) and macrophages, molecules such as CD71 (receptor for transferrin), Km8, and 2SF9 are found preferentially on mature tissue macrophages, including AMs [12-14], thus representing markers of the final stage of differentiation.

Distinct phenotypic subpopulations of AMs have recently been identified [15, 16]. Macrophages with properties of dendritic, phagocytic or suppressive cells may be separated on the basis of the co-expression of membrane antigens RFD1, RFD7 and RFD9 [17, 18]. Other subpopulations of AMs can be distinguished by monoclonal antibodies against 27E10 molecule (marker of the acute phase inflammatory macrophages) and RM3/1 (marker of late phase inflammatory macrophages) [19, 20].

Several recently characterized membrane molecules of AMs are the focus of interest as prospective target molecules for possible immunomodulation of pathological processes in the lung. In particular, the CD14 antigen, a receptor for the lipopolysaccharide (LPS) binding protein, representing an important trigger for tumour necrosis factor-α (TNF-α) production, seems to be promising in this respect [21, 22].

Since the phenotype of AMs has been shown to be modulated during pathological reactions in the lung parenchyma [23, 24], we hypothesized that the expression of AM
membrane antigens may vary depending on the type of immune or immunopathological reactions in the lung. In this respect, the purpose of this study was to characterize the AM phenotype in relation to changes in the proportions of different types of immune cells and in the CD4/CD8 ratio in bronchoalveolar lavage (BAL).

Methods

Study population

The AM phenotype was studied in 76 consecutive subjects (24 being smokers and 16 treated with corticosteroids). There were 32 patients with sarcoidosis, 17 of these with active disease according to previously described clinical criteria [25]. Among those with active sarcoidosis, 8 were type 1 and 9 were type II disease, 8 cases were studied at the first presentation of disease, one patient was treated with steroids, and one was a smoker. Furthermore, the study population included 8 patients with idiopathic pulmonary fibrosis (IPF), 7 patients with chronic bronchitis, 6 with bacterial pneumonia, and 9 with pneumoconiosis (3 with silicosis and 6 with asbestosis). As controls, 14 subjects (9 smokers, one treated with steroids) with no evidence of interstitial lung disease were studied. Nine of the controls were undergoing routine bronchoscopy for suspected lung cancer (1 case with peripheral lung cancer, 1 case with tracheal polyposis and 7 patients without final evidence of lung disease), 2 subjects had a control BAL performed several months following viral pneumonia and 3 were normal healthy volunteers. In all of these 14 controls, the lung lavaged was roentgenographically normal, and BAL cytology showed a normal differential count (table 1).

For the comparison of AM phenotype with the different BAL profiles, patients were divided into three groups: Group 1) those having the proportion of AMs higher than 85%, lymphocytes lower than 15%, and granulocytes lower than 3% (31 subjects); Group 2) subjects with the proportion of BAL lymphocytes higher than 30% (29 subjects); and Group 3) those having more than 10% of neutrophils and/or eosinophils in BAL (6 subjects). An increase in the percentage of both lymphocytes and granulocytes above the aforementioned levels was found in only 3 cases, this group being too small for statistical evaluation.

Bronchoalveolar lavage

Bronchoalveolar cells were sampled from the air spaces of the lung using BAL, performed by instillation of a total of 100 ml 0.9% saline solution in five 20 ml aliquots into the middle lobe or the lingula, with immediate aspiration after each aliquot. Informed consent for the procedure was obtained from all subjects. After filtration through two layers of gauze, the recovered fluid was centrifuged (10 min, 500 x g), and the cells were counted in a haemocytometer. Differential counts were made from smears stained with May-Grünwald-Giemsa (at least 300 cells were counted). A trypan blue exclusion test for cell viability was performed.

Immunocytochemical analysis

Bronchoalveolar cells were characterized by use of 15 monoclonal antibodies against various myeloid antigens (table 2) and 3 monoclonal antibodies for immunotyping of T-lymphocytes (OKT3, OKT4 and OKT8, all from Ortho Diagnostics). Membrane antigens were identified by the peroxidase-antiperoxidase method performed on adhesion slides (Bio-Rad) [42]. Briefly, 10 µl cell suspension, at a concentration of 5 x 10⁶ cells·ml⁻¹, was added to each of the reaction areas. After 10 min, the cells were settled and firmly attached to the slide. After the incubation of cells with monoclonal antibodies, fixation with glutaraldehyde (0.05%) for 5 min was performed. In the case of 25F9 and 27E10, the cells were fixed with 80% acetone-ethanol before incubation with monoclonal antibodies. A preincubation with a gelatine containing medium supplemented by 10% swine serum was performed to reduce nonspecific binding of immunoglobulins to glass and cells. Next, rabbit antimouse immunoglobulin (Dakopatts) was added, followed by swine antirabbit immunoglobulin (Dakopatts) and peroxidase-antiperoxidase immunocomplex from rabbit (Dakopatts). Diaminobenzidine (Sigma) was used as substrate, and OsO₄ (Sigma) for the postfixation. To evaluate the reaction, the slides were viewed under a light microscope. Two hundred cells were counted in each reaction area.

Table 1. - Basic data of BAL cytology in different groups of patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Total cells ×10⁶</th>
<th>AMs</th>
<th>Lymph</th>
<th>Neut</th>
<th>Eosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>14</td>
<td>13±10</td>
<td>9±6</td>
<td>7±6</td>
<td>2±2</td>
<td>0.3±0.6</td>
</tr>
<tr>
<td>Inactive sarcoidosis</td>
<td>15</td>
<td>11±7</td>
<td>6±5</td>
<td>30±19</td>
<td>2±2</td>
<td>0.1±0.5</td>
</tr>
<tr>
<td>Active sarcoidosis</td>
<td>17</td>
<td>12±7</td>
<td>46±15</td>
<td>51±16</td>
<td>2±2</td>
<td>0.9±2.0</td>
</tr>
<tr>
<td>IPF</td>
<td>8</td>
<td>2±8</td>
<td>71±25</td>
<td>17±24</td>
<td>9±13</td>
<td>2.1±2.0</td>
</tr>
<tr>
<td>Chronic bronchitis</td>
<td>7</td>
<td>8±5</td>
<td>84±14</td>
<td>8±7</td>
<td>7±14</td>
<td>0.3±0.8</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>6</td>
<td>8±11</td>
<td>40±27</td>
<td>49±30</td>
<td>9±28</td>
<td>2.3±3.5</td>
</tr>
<tr>
<td>Pneumococcosis</td>
<td>9</td>
<td>18±12</td>
<td>75±26</td>
<td>20±24</td>
<td>4±25</td>
<td>0.8±1.1</td>
</tr>
</tbody>
</table>

BAL: bronchoalveolar lavage; AMs: alveolar macrophages; Lymph: lymphocytes; Neut: neutrophils; Eosin: eosinophils; IPF: idiopathic pulmonary fibrosis.
Table 2. - Antigens determined in this study

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Source</th>
<th>Antigen References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOT 16</td>
<td>Dianova</td>
<td>LFA-1 (CD11a)</td>
</tr>
<tr>
<td>OKM1</td>
<td>Ortho Diagn.</td>
<td>Mac-1, CR3 (CD11b)</td>
</tr>
<tr>
<td>KIM1</td>
<td>Behring</td>
<td>p150/95, CR4 (CD11c)</td>
</tr>
<tr>
<td>IOT 18</td>
<td>Dianova</td>
<td>β-chain of β2-integrins (CD18)</td>
</tr>
<tr>
<td>ICAM</td>
<td>Dianova</td>
<td>ICAM-1 (CD54)</td>
</tr>
<tr>
<td>OKT9</td>
<td>Ortho Diagn.</td>
<td>Transferrin receptor (CD71)</td>
</tr>
<tr>
<td>25F9</td>
<td>IED Münster</td>
<td>Marker of mature Mo</td>
</tr>
<tr>
<td>KIM8</td>
<td>Behring</td>
<td>Marker of phagocytic Mo</td>
</tr>
<tr>
<td>RFD1</td>
<td>RFH London</td>
<td>Marker of dendritic cells</td>
</tr>
<tr>
<td>RFD7</td>
<td>RFH London</td>
<td>Marker of subpopulation of mature Mo</td>
</tr>
<tr>
<td>RFD9</td>
<td>RFH London</td>
<td>Marker of epitheloid cells</td>
</tr>
<tr>
<td>27E10</td>
<td>IED Münster</td>
<td>Marker of inflammatory acute phase Mo</td>
</tr>
<tr>
<td>RM3/1</td>
<td>IED Münster</td>
<td>Marker of inflammatory late phase Mo</td>
</tr>
<tr>
<td>OKM14</td>
<td>Ortho Diagn.</td>
<td>Receptor for LPS-BP (CD14)</td>
</tr>
<tr>
<td>KIM2</td>
<td>Behring</td>
<td>Myeloid antigen</td>
</tr>
</tbody>
</table>

RFH: Royal Free Hospital; IED: Institute of Experimental Dermatology; LFA-1: lymphocyte function associated antigen; ICAM-1: intercellular adhesion molecule; Mo: macrophage; LPS-BP: lipopolysaccharide binding protein.

Statistics

The data are shown as mean±so. Spearman’s rank coefficient of correlation was determined and tested for statistical significance. For the comparison of different groups of patients, one way analysis of variance (ANOVA) test was used. A p value of <0.05 was considered significant.

Results

Adhesion associated molecules

CD11a (α-chain of lymphocyte function associated antigen-1 (LFA-1) molecule) was expressed on the majority of alveolar macrophages (mean percentage 92±7%) without any significant differences between the groups studied. There was no correlation with the different cell types.

CD11b (Mac-1, C3R) expression on alveolar macrophages correlated positively with the total cell count (r=0.296, p=0.011) and the percentage of lymphocytes (r=0.523, p=0.0001) in BAL fluid. A significant increase of the antigen expression was found in patients with active sarcoidosis (83±11%) compared to control subjects (39±20%) (p<0.001), patients with chronic bronchitis (40±26%) (p<0.01), and patients with inactive sarcoidosis (57±15%) (p<0.05).

Antigens characteristic for subpopulations of macrophages

CD71 (transferrin receptor) expression on AMs correlated with the percentage of bronchoalveolar lymphocytes (r=0.592, p=0.0001), and the CD4/CD8 ratio of T-lymphocytes in BAL (r=0.348, p=0.017). The percentage of CD71 positive AMs was increased in patients with active sarcoidosis (93±12%) compared to control subjects (78±10%) (p<0.01).

25F9 antigen was uniformly expressed in the majority of AMs (mean 90±10%) and correlated inversely with the percentage of bronchoalveolar neutrophils (r=-0.277, p=0.035) and eosinophils (r=-0.308, p=0.019). Corticosteroid treatment was associated with the decrease in 25F9 expression (r=-0.279, p<0.05).

KIM8 antigen membrane expression appeared to show a relationship with the percentage of bronchoalveolar lymphocytes (r=0.470, p=0.0001) and neutrophils (r=0.296, p=0.011). Other correlations did not reach statistical significance.

Antigens characteristic for subpopulations of macrophages

RFD1 (marker of dendritic cells) expression correlated directly with the percentage of bronchoalveolar lymphocytes (r=0.396, p=0.001), the proportion of CD4+ T-lymphocytes (r=0.425, p=0.006), and the CD4/CD8 ratio (r=0.405,
and with a subpopulation of mature macrophages) was detected preferentially on medium size AMs. The antigen expression correlated positively with the percentage of bronchoalveolar lymphocytes (r=0.441, p=0.0002).

RFD7 (marker of epitheloid cells and macrophages of germinal centres) expression on AMs correlated with the percentage of bronchoalveolar lymphocytes (r=0.469, p=0.0001). A negative correlation was found with the proportion of bronchoalveolar eosinophils (r=-0.257, p=0.031). A significant increase in RFD9 expression was found in patients with active sarcoidosis (71±19%) in comparison to patients with idiopathic pulmonary fibrosis (34±21%) (p<0.05) and pneumoconiosis (36±27%) (p<0.05).

27E10 (marker of inflammatory acute phase macrophages) was detected in small macrophages and in neutrophils. The percentage of 27E10 positive AMs correlated positively with the relative proportion of neutrophils (r=0.364, p=0.006). Although these macrophages were seen most frequently in patients with pneumonia (21±20%), the difference to other groups of patients did not reach statistical significance.

RFD9 (marker of epitheloid cells and macrophages of germinal centres) expression on AMs correlated with the percentage of bronchoalveolar lymphocytes (r=0.462, p=0.0003) without any significant difference between the groups studied.

Other myeloid antigens

CD14 (receptor for LPS binding protein) was detected on AMs in relationship with the percentage of bronchoalveolar lymphocytes (r=0.434, p=0.0002).

KiM2 antigen expression correlated with the percentage of bronchoalveolar lymphocytes (r=0.640, p=0.0001). KiM2 positive AMs were more frequently found in patients with active sarcoidosis (mean 85±8%) compared to control subjects (57±24%) (p<0.05).

Comparison of three groups of patients selected by differential cell counts of BAL cells (figs 1-3)

The group of subjects with evident lymphocytosis in BAL fluid (Group 2: lymphocytes >30%) had significantly higher expression of 11 out of 15 AM membrane antigens (all but CD11a, CD11c, CD18, 25F9) in comparison to subjects with normal differential count (Group 1: lymphocytes <15%, granulocytes <5%). Patients having a markedly increased percentage of granulocytes in BAL (Group 3: proportion of neutrophils and/or eosinophils >10%) differed from the subjects with normal BAL count in the higher expression of 27E10 antigen (p<0.01).

Relationship between different membrane antigens expressed on AM

In order to evaluate how the expression of the different membrane antigens correlated to each other, 105 bilateral coefficients of correlation were determined, 59 of them being significant. The expression of antigens 25F9, CD18 and CD11c did not correlate with the expression of most other membrane antigens. Thus, these three antigens seem to be rather constitutively expressed.

Effect of smoking on AM phenotype

Within the control group (8 smokers versus 6 non-smokers) there were no differences in the expression of AM membrane antigens (p>0.05). The effect of smoking was not independently evaluated in the 16 smokers with different diagnoses, since the groups were too small for this statistical evaluation.

Fig. 1. - The proportion (mean±SD) of AMs from subjects with normal BAL count (Group 1; n=31), lymphocytic alveolitis (Group 2; n=29), and with increased proportions of BAL neutrophils (Group 3; n=6) expressing adhesion molecules CD11a, CD11b, CD11c, CD18 and CD54. The expression of CD11b and CD54 is significantly increased in patients with lymphocytic alveolitis as compared with subjects with normal BAL (p<0.05 and p<0.001, respectively) and with neutrophilic alveolitis (p<0.05 and p<0.001, respectively). □: Group 1; normal BAL count; □□: Group 2; BAL lymphocytes >30%. □□□: Group 3; BAL neutrophils >10%; AMs: alveolar macrophages; BAL: bronchoalveolar lavage.
Discussion

Membrane antigens of human alveolar macrophages are essential for their ability to modulate immune and inflammatory processes in the lung. The present study extends former observations on the AM phenotypes by analysing a large panel of monoclonal antibodies for the simultaneous determination of 15 myeloid antigens, and comparing the immunocytochemical results with the relative proportions of BAL cells.

Our data demonstrated significant phenotypic changes of AMs associated with a lymphocytic predominance of bronchoalveolar cells. The observed up-regulation of adhesive molecules involved in cell-cell communications, especially of CD54 (ICAM-1), may be related to the higher antigen-presenting activity of AMs described previously in diseases with a lymphocytic alveolitis [43, 44]. Also, the increased expression of RFD1 antigen, a unique epitope of human leucocyte antigen-DR (HLA-DR) molecules, was found to be associated with a lymphocytic alveolitis. A recent paper reported that the percentage of RFD1+D7+ macrophages was directly proportional to the lymphocytes present [4].

The expression of the CD14 molecule, known to be an
important trigger for TNF-α production [45], also showed a strong relationship with the proportion of BAL lymphocytes. TNF-α, a cytokine with potent local and systemic effects [46], has been found to be secreted at higher levels by AMs of patients with pulmonary sarcoidosis [47], and tuberculosis [48], diseases with a characteristic lymphocytic alveolitis [49, 50]. Whether the recently reported dexamethasone or interleukin-4 (IL-4) induced down-regulation of TNF-α release by activated AMs [51, 52] is also associated with a down-regulation of CD14 and other molecules on the AM membrane should be investigated in further studies.

Our analysis of the expression of transferrin receptors (CD71) and RFD9 antigens by AMs confirmed the previously described increase in several forms of active interstitial lung diseases [23, 24, 53]. The role of these molecules, as well as the function of the antigens KiM8, KiM2, and RM3/1, in the aetiopathogenesis of immunopathological reactions of the lung remains hypothetical.

A predominance of bronchoalveolar eosinophils was not associated with the enhanced expression of AM membrane antigens, and even correlated inversely with the expression of RFD1, RFD9 and 2SF9. In pathologic processes associated with a higher number of BAL neutrophils, 27E10 positive small AMs were more frequently found. This molecule is also expressed on neutrophils, and may be of particular importance in the acute phase of inflammation [19]. The 27E10 epitope, the heterodimer of two migration inhibitory factor-related proteins, MRP8 and MRP14, is characteristic for macrophages spontaneously releasing IL-1β, TNF-α and IL-6 [54] and frequently found in acute inflammatory tissues, but largely or completely absent in chronic inflammation [55]. Furthermore, a higher membrane expression of the KiM8 molecule, which we found to be present intracellularly in almost all AMs (own unpublished data), correlated with the proportion of both bronchoalveolar neutrophils and lymphocytes. This molecule is closely associated with cytoplasmic vesicles [13], and its expression may reflect the phagocytic ability of AMs.

The relationship between the AM phenotype and the different proportions of BAL cells was proved both by correlating individual AM antigens the percentage of lymphocytes or granulocytes, and by comparing the AM phenotypic patterns in three different groups of patients selected by their alveolitis profile of bronchoalveolar cells. The expression of most membrane antigens of AM correlated with each other. The most stable markers of AM seem to be the antigens 2SF9, CD18 and CD11c, the expressions of which did not show prominent changes in comparison to the other antigens.

Since cigarette smoking has been reported to be a factor influencing the composition of AM surface proteins [56], we also evaluated the correlation between smoking habit and cell phenotype in our control group, and found no significant difference in the AM phenotype between smokers and nonsmokers. The number of smokers in the various disease categories was too small to allow a separate comparison between smokers and nonsmokers for the different diseases. Corticosteroid therapy was not associated with profound changes of the AM phenotype. We could not confirm the enhancing effect of corticosteroids on RM3/1 antigen expression reported for blood monocytes [37], or on RFD7 for cultured AMs, and the suppressing effect on RFD1 antigens [35].

Only rarely, changes in the phenotype of AMs could be related to a specific diagnosis. Prominent changes of AM phenotype were found in patients with active sarcoidosis, a disorder characterized by a lymphocytic alveolitis, with more frequent expression of the molecules CD54, KiM2, CD71, CD11b and RFD9. A more detailed analysis of AM membrane antigen expression in sarcoidosis patients has been published in our recent reports [10, 57].

In conclusion, our study indicates that the expression of AM membrane antigens is related to the type of immune or immunopathological reaction in the lung. Although these phagocytes represent an extremely heterogeneous cellular population, their phenotype probably reflects different steps of defensive or pathologic processes in the lung. Similarities in the alveolar macrophage response to different stimuli may limit the diagnostic usefulness of AM phenotyping but might contribute to better prediction of the clinical course in some lung diseases. In this respect, further studies correlating the expression of selected AM membrane antigens with the clinical course are needed.

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