Intercellular adhesion molecule 1 (ICAM-1) in the pathogenesis of mononuclear cell alveolitis in pulmonary sarcoidosis

Klaus Dalhoff, Sabine Bohnet, Jörg Braun, Burkhard Kreft, Karl J Wießmann

Abstract

Background—Alveolitis in pulmonary sarcoidosis is characterised by an accumulation of highly activated macrophages and CD4+ lymphocytes in the alveolar compartment. The role of intercellular adhesion molecule 1 (ICAM-1) expression on alveolar cells has been studied in this context.

Methods—Using a sandwich ELISA technique, ICAM-1 expression on alveolar macrophages from 17 consecutive untreated patients with pulmonary sarcoidosis and six healthy normal volunteers was quantified. In addition, parameters of macrophage activation (tumour necrosis factor α (TNFα) and superoxide anion release) were evaluated.

Results—Significantly elevated expression could be demonstrated on alveolar macrophages from patients with pulmonary sarcoidosis compared with healthy controls (mean (SD) 0-74 (0-24) ELISA units (EU) v 0-46 (0-12) EU). On subdividing the patients into those with active and those with inactive disease, only the former showed increased ICAM-1 levels on alveolar macrophages (0-82 (0-27) EU) compared with control alveolar macrophages. No differences were detected in serum levels of soluble ICAM-1 between patients and controls. ICAM-1 expression on alveolar macrophages from patients with sarcoidosis correlated with the spontaneous release of TNFα but not with the release of the superoxide anion by the activated macrophages. There was no correlation with the percentage of lymphocytes or the absolute number of CD4+ cells in bronchoalveolar lavage fluid.

Conclusions—Increased ICAM-1 surface expression on alveolar macrophages reflects disease activity in the pulmonary compartment. Considering the significance of adhesion molecules during antigen presentation and lymphocyte activation, ICAM-1 expression on alveolar macrophages may have an important role in the immune process of pulmonary sarcoidosis.

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staining, we determined ICAM-1 expression in a group of untreated patients with pulmonary sarcoidosis and a group of healthy volunteers.

**Methods**

**STUDY POPULATION**

The study population consisted of 17 consecutive untreated patients with biopsy proven pulmonary sarcoidosis (radiological type 0, n = 1; type I, n = 9; type II, n = 7). There were 10 patients with active and seven with inactive disease. The assessment of clinical disease activity was performed according to previously published criteria. Briefly, new or increasing respiratory or systemic symptoms, a reproducible decline in vital capacity and/or diffusing capacity of >10%, and new or progressing infiltrates in the chest radiograph were accepted as signs of active disease (table 1). Six healthy volunteers were studied as controls. There were no smokers in either group which is notable as smoking influences expression of adhesion molecules. Ethical committee approval and individual informed consent were obtained. Clinical data and bronchoalveolar lavage cell counts are summarised in table 2.

**BRONCHOALVEOLAR LAVAGE AND CELL ISOLATION**

Bronchoalveolar lavage was performed from the middle lobe using a flexible fibreoptic bronchoscope under standard conditions. The lavage volume was 200 ml with a mean recovery of 84%, which was similar in patients and controls. The first 20 ml aliquot of the lavage fluid which is known to be contaminated by bronchial secretions was discarded and aliquots were pooled. A total cell count was made by a haemocytometer using a haemocytometer.

<table>
<thead>
<tr>
<th>Active</th>
<th>Inactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chest radiograph</td>
<td></td>
</tr>
<tr>
<td>Type 0 progressing stable</td>
<td>0</td>
</tr>
<tr>
<td>Type I progressing stable</td>
<td>1</td>
</tr>
<tr>
<td>Type II progressing stable</td>
<td>2</td>
</tr>
<tr>
<td>Lung function*</td>
<td></td>
</tr>
<tr>
<td>Vital capacity decreasing stable</td>
<td>5</td>
</tr>
<tr>
<td>Diffusing capacity decreasing stable</td>
<td>5</td>
</tr>
<tr>
<td>Respiratory symptoms</td>
<td>7</td>
</tr>
<tr>
<td>Systemic symptoms</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>5</td>
</tr>
<tr>
<td>Erythema nodosum</td>
<td>4</td>
</tr>
<tr>
<td>Arthritis/arthralgia</td>
<td>8</td>
</tr>
<tr>
<td>Exothoracic sarcoidosis</td>
<td>1</td>
</tr>
<tr>
<td>Uveitis</td>
<td>1</td>
</tr>
<tr>
<td>Skin involvement</td>
<td>1</td>
</tr>
</tbody>
</table>

*Data available only for 16 patients.

(Neubauer), then lavage cell differentials were determined by Wright-Giemsa stain of cytospin preparations (Cytops II, Shandon). Cell viability was assessed using trypan blue dye exclusion. For differentiation of lymphocyte subsets (CD3, CD4, CD8) fluorescein conjugated monoclonal antibodies (Ortho Diagnostics, Beerse, Belgium) were used. Lavage cells were washed twice with phosphate buffered saline and resuspended at a concentration of 1 × 10⁶ alveolar macrophages/ml in M199 supplemented with fetal calf serum (5%, Gibco), L-glutamine (2 mmol/l, Gibco), and penicillin/streptomycin (1 mg/ml, Gibco).

**ICAM-1 EXPRESSION ON ALVEOLAR MACROPHAGES AND LYMPHOCYTES**

ICAM-1 expression on alveolar macrophages was determined by a sandwich ELISA technique. Mononuclear lavage cells containing viable alveolar macrophages at a density of 1 × 10⁶/ml were seeded into 96-well flat bottom microtitre plates (Greiner, Nürtingen, Germany) for three hours (37°C, 5% carbon dioxide), allowing the macrophages to adhere. Non-adherent cells were removed by gentle washing and serum-supplemented M199 was added. Incubation was continued for 16 hours at 37°C in a total humidified atmosphere containing 5% carbon dioxide. Cells were then fixed with 1% paraformaldehyde (one hour, 21°C) and free binding sites were blocked by adding a 2% solution of bovine serum albumin (BSA) diluted in phosphate buffered saline (PBS, 0-1 mol/l, pH 7.2) for one hour (37°C). The monoclonal mouse anti-ICAM-1 antibody (clone 84H10, Dianova, Hamburg, Germany; 1:1000 in 0.1% BSA/PBS) was added for two hours at 37°C. Peroxidase conjugated goat antimouse antibody (Sigma, Deisenhofen, Germany, 1:1000 in 0.1% BSA/PBS) was used as developing antibody. Finally, ABTS (Sigma, 1 mg/ml) and hydrogen peroxide (2-8 µl, 30% H₂O₂/10 ml) in 0-1 mol/l sodium acetate (pH 4.2) was added. The colour was allowed to develop for 120 minutes and plates were read with a microplate reader (Behring EL 311) 405 nm.

Results were expressed as ELISA units (EU) from quadruplicate wells after subtraction of non-specific background absorbance which was determined in the absence of the
primary antibody. The number of adherent cells at the time of the ELISA was assessed in parallel wells by crystal violet staining. Using this method we found no differences between the three groups of our study (data not shown).

In addition, ICAM-1 expression on bronchoalveolar cells was determined by immunocytochemical staining on cytocentrifuge preparations using the alkaline phosphatase monoclonal mouse antialkaline phosphatase (APAAP) complex as described.20

SOLUBLE ICAM-1 (sICAM-1) SERUM LEVELS
Serum was collected at the time of bronchoalveolar lavage and frozen at −20°C. sICAM-1 levels were determined with a commercially available kit (Serva, Heidelberg, Germany).

TNFα BIOASSAY
Tumour necrosis factor α (TNFα) activity was measured with an L929 fibroblast lytic assay as previously described.21 Briefly, L929 cells (6 × 104 well) were cultured in 96-well flat bottom microtitre plates (Nunc) containing serial dilutions of conditioned supernatant of sarcoid alveolar macrophages in the presence of actinomycin D. After 20 hours of incubation the remaining cells were stained with crystal violet. The TNFα concentration of the samples was quantified by comparing the results with the linear portion of a standard curve obtained with recombinant TNFα (Serva, Heidelberg, Germany, specific activity 6-6 × 10⁴ units/mg). The specificity of this bioassay was tested by neutralising peak samples with a goat antihuman TNFα antibody (obtained through H Biermann, Bad Nauheim, Germany from British Biotechnology).

SUPEROXIDE ANION
The production of superoxide anion was determined by a ferricytochrome c microassay.22 Briefly, 10⁶ alveolar macrophages were incubated in a 96-well microtitre plate with 100 μmol ferricytochrome c ± 0·2 mg/ml superoxide dismutase, with or without 10 mmol sodium fluoride or 100 nmol PMA. All experiments were done in triplicate. Optical density was determined at 550 nm over a period of two hours at 30 minute intervals on a microplate reader.

STATISTICS
Non-parametric statistics were used throughout the study. The Mann–Witney U test was used for independent samples. Correlations were made with the Spearman rank correlation, using the median as a marker of central tendency. Differences were considered significant at p < 0·05.

Results
TOTAL AND DIFFERENTIAL CELL COUNT
Table 2 shows the cell counts in bronchoalveolar lavage fluid. The total number of cells recovered from the bronchoalveolar lavage fluid was increased in patients with pulmonary sarcoidosis. Furthermore, the percentage of lymphocytes was significantly increased compared with the healthy controls (p < 0·005).

ICAM-1 ON ALVEOLAR CELLS AND SERUM LEVELS OF sICAM-1
ICAM-1 expression could be detected on alveolar macrophages in all cases (fig 1). Significantly elevated expression was seen on alveolar macrophages from patients with sarcoidosis compared with controls (mean (SD) 0·74 (0·24) EU v 0·46 (0·12) EU, p < 0·05). Only patients with apparently active disease showed significantly increased ICAM-1 levels on alveolar macrophages (0·82 (0·27) EU) compared with alveolar macrophages from the controls.

Immunocytochemical staining confirmed the results of the ELISA for the macrophage population in the bronchoalveolar lavage fluid. Using the APAAP complex, ICAM-1 expression could also be demonstrated on alveolar lymphocytes from patients with sarcoidosis (fig 2).

Serum levels of sICAM-1 ranged from 200 to 700 ng/ml and were similar in patients and controls (490 (130) ng/ml v 320 (90) ng/ml).
No correlation between sICAM-1 levels and ICAM-1 surface expression on alveolar macrophages was demonstrated.

RELEASE OF TNFα AND OXIDANTS BY ALVEOLAR MACROPHAGES

All alveolar macrophages from patients with sarcoidosis spontaneously released TNFα and free oxygen radicals whereas only a small amount of activity was detected in the supernatants of controls (table 3). TNFα release of alveolar macrophages correlated strongly with the ICAM-1 surface expression of those cells \((r = 0.69, p < 0.05; \text{fig 3})\). The spontaneous release of free oxygen radicals was significantly elevated in patients and did not differ between subjects with active and inactive disease (table 3). No correlation with ICAM-1 expression could be shown.

The intensity of the lymphocytic alveolitis as assessed by absolute numbers and percentage of CD4+ lymphocytes in bronchoalveolar lavage fluid was not related to the enhanced expression of ICAM-1 on alveolar macrophages.

Discussion

Pulmonary sarcoidosis is an inflammatory disorder of unknown aetiology, characterised by a mononuclear cell alveolitis and non-caseating granulomas, leading in some cases to interstitial fibrosis.\(^{24-26}\) To elucidate the possible role of adhesion molecules in this disease, we investigated the contribution of ICAM-1 to the process of recruitment and activation of inflammatory cells in the lung.

ICAM-1 levels on alveolar macrophages were elevated in patients with pulmonary sarcoidosis. This increase was statistically significant only in patients with active disease. No other statistical difference between the two groups of patients could be established, which is probably due to the limited number of subjects in our study.

There are several possible mechanisms leading to the increased ICAM-1 levels we measured. One could be enhanced recruitment of blood monocytes into the pulmonary compartment. In a recent study by Melis et al., however, no relation between ICAM-1 expression and different blood monocyte associated markers on alveolar macrophages was found.\(^{15}\) Furthermore, they found no difference between ICAM-1 expression on blood monocytes from patients with sarcoidosis and from healthy volunteers. This is in line with our findings that serum levels of sICAM-1 were similar in our patients and controls.

TNFα activity in the supernatant of alveolar macrophages correlated strongly with ICAM-1 surface expression of those cells. It has been shown previously that TNFα and other cytokines induce ICAM-1 on endothelial cells, whereas airway epithelial cells are predominantly responsive to \(\tau\)-interferon.\(^{27}\) We recently showed that TNFα induces ICAM-1 on control alveolar macrophages in vitro (unpublished observation). Thus, it seems likely that in sarcoidosis ICAM-1 expression on alveolar macrophages is regulated in an autocrine manner, supporting the concept that alveolar macrophages are pivotal cells in the pathogenesis and maintenance of the inflammatory process in the pulmonary compartment. Further support for a local upregulation of ICAM-1 was that serum levels of sICAM-1 were not related to the activation demonstrated on alveolar macrophages.

Few studies exist which describe elevated ICAM-1 levels on alveolar macrophages in patients with sarcoidosis.\(^{11,16}\) However, to our knowledge this is the first description of increased ICAM-1 expression in correlation with other parameters of immunological activity. We found no correlation between upregulated ICAM-1 expression of alveolar macrophages to the number of CD4+ lymphocytes in bronchoalveolar lavage fluid, which is in agreement with previous work.\(^{15}\) Elevated ICAM-1 surface expression was not restricted to alveolar macrophages in patients with sarcoidosis, however, but could also be demonstrated on alveolar lymphocytes.\(^{28}\) This suggests that the interaction between ICAM-1 and its ligands on macrophages and CD4+ lymphocytes functions reciprocally.

### Table 3 Mean (range) data of macrophage activation in bronchoalveolar lavage fluid of patients with active and inactive sarcoidosis and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Total</th>
<th>Active</th>
<th>Inactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1 (EU/10⁵ AM)</td>
<td>0.46 (0.29-0.57)</td>
<td>0.78 (0.42-1.17)</td>
<td>0.82 (0.44-1.17)</td>
<td>0.64 (0.42-0.84)</td>
</tr>
<tr>
<td>TNFα (units/10⁶ AM)</td>
<td>3 (0-10)</td>
<td>239 (5-1345)</td>
<td>319 (6-1345)</td>
<td>59 (5-141)</td>
</tr>
<tr>
<td>O₂⁻ (nmmol/10⁸ AM)</td>
<td>0.76 (0-1-40)</td>
<td>2.59 (0-37-479)</td>
<td>2.52 (1-0-479)</td>
<td>2.75 (0-37-5-97)</td>
</tr>
</tbody>
</table>

ICAM-1—intercellular adhesion molecule 1; EU—ELISA unit; TNFα—tumour necrosis factor α; O₂⁻—superoxide anion; AM—alveolar macrophage.
Considering the strong correlation we found between ICAM-1 expression and TNFα secretion of the activated macrophages, we conclude that upregulated ICAM-1 levels reflect disease activity in the pulmonary compartment. The fact that we could not show a correlation with the number of CD4+ lymphocytes agrees with other studies, suggesting different mechanisms of activation for the lymphocytic and macrophage component of the mononuclear cell infiltrates in pulmonary sarcoidosis. 11,24

It is not clear whether upregulated ICAM-1 expression on alveolar macrophages is an early event during the development of the mononuclear cell infiltrate or a result of the inappropriate cytokine secretion of alveolar macrophages and lymphocytes in the pulmonary compartment. Further research is needed to characterise the exact position of ICAM-1 in the inflammatory cascade in sarcoidosis.

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