Elevated Level of Soluble HLA Class I Antigens in Serum and Bronchoalveolar Lavage Fluid in Patients with Sarcoidosis

Nobuo Ogisu, Shigeki Sato, Haruhiko Kawaguchi, Yoshiki Sugihara, Toshiyuki Mori, Takashi Niimi, Hiroyoshi Maeda, Kenji Akita, Yuka Yamada and Ryuzo Ueda

Abstract

Objective Soluble HLA class I antigens (sHLAs) in human serum have been reported to be associated with allografts and autoimmune disease and could modify immunological reactions induced by membrane type HLAs. To investigate the clinical significance of sHLAs in sarcoidosis, we assessed concentrations of sHLAs in both serum and bronchoalveolar lavage fluid (BALF) and also examined their production by peripheral blood mononuclear cells (PBMCs) and BALF cells.

Methods Concentrations of sHLAs were determined by enzyme-linked immunosorbent assay, using a monoclonal antibody against HLA class I (W6/32) and an enzyme-labeled polyclonal antibody to human β2-microglobulin. PBMCs and BALF cells were cultured in the presence or absence of either LPS or PHA.

Patients Serum levels of sHLAs were assessed in 96 patients with sarcoidosis and in 32 healthy control subjects. sHLAs concentrations in BALF were also investigated in 17 active sarcoidosis patients and in 13 control subjects.

Results sHLAs levels in both serum and BALF were higher in sarcoidosis cases than in control subjects (p<0.05, in both). In the patients, values were significantly higher in active than in inactive stages (p<0.001) and significantly correlated with angiotensin-converting enzyme (ACE) levels. Both PBMCs and BALF cells produced enhanced amounts of sHLAs in patients with active sarcoidosis compared with those in control subjects.

Conclusion These results demonstrated that the level of sHLAs in serum is a useful index of disease activity of sarcoidosis, partly reflecting production by PBMCs and BALF cells.

Key words: active sarcoidosis, disease activity, angiotensin-converting enzyme, enzyme-linked immunosorbent assay

Introduction

HLA class I antigens, heterodimeric cell surface antigens composed of a 44 kDa highly polymorphic heavy chain and noncovalently associated 12 kDa β2 microglobulin (1), play an important role in the immune recognition of antigens, response to organ transplants, and auto-immune disease. Soluble HLA class I antigens (sHLAs) found in normal human serum were first shown by Rood to inhibit anti-HLA sera (2) and modify the immunological reactions induced by membrane type HLA. Especially in liver transplantation, it has been reported that sHLAs released by liver parenchymal cells could induce tolerance against host rejection and monitoring of these antigens may be a useful indicator of graft pathology and function (3, 4). Furthermore, elevation of sHLAs has been reported to be associated with some viral systemic infections, including cases with human immunodeficiency virus (5) and hepatitis virus (6).

Sarcoidosis is a disease of unknown etiology characterized by the presence of noncaseating epithelioid cell granulomas in systemic organs, and several immunological disorders (7, 8). Recent investigations into its pathogenesis have demonstrated that activated T cells at sites of inflammation are oligoclonal, consistent with a conventional antigen-driven T cell immune response (9–11). Considering the high serum levels of sHLA in association with an activated immune response, we considered that these levels in patients with sarcoidosis might be of interest. In this study, we therefore examined whether sHLA concentrations in sera and bronchoalveolar lavage fluid (BALF) from patients may be useful for the evaluation of disease activity. We also demonstrated that peripheral blood mononuclear cells (PBMCs) and BALF cells derived from active sarcoidosis patients release enhanced amounts of sHLAs compared with those from healthy controls.

Materials and Methods

Study population (Table 1)
The serum of a total of 94 sarcoidosis patients, 37 men and...
57 women with a mean (SD) age of 51.4±15.4 years, was evaluated for levels of sHLAs along with that from 32 individuals, 19 men and 13 women with a mean age of 50.6±15.3 years, as normal subjects. Sarcoidosis was diagnosed based on the clinical picture and the presence of epithelioid cell granulomas in biopsy specimens from lung, skin, or lymph nodes (12). According to the classification system defined by DeRemee (13), there were 70 patients in roentgenographic stage I, 15 in stage II, and nine in stage III. Further, 53 patients were defined as having active disease since they had newly developed chest radiological abnormalities and clinical symptoms, or had a deterioration in chest radiographic findings and clinical status during the previous 3 months (14). The others were considered to have inactive disease. None of the patients was receiving any corticosteroids. In this study population, 20 of patients with active sarcoidosis and 10 control subjects were examined for production of sHLAs from PBMCs. In 17 active sarcoidosis patients and 13 healthy control subjects, bronchoalveolar lavage (BAL) was performed as described previously (15). Both serum and BALF were stored at −30°C before being evaluated for the sHLAs concentration.

All subjects had no history of cardiopulmonary or other medical illness including tuberculosis, infection, and autoimmune diseases. All the patients for which BAL was analyzed had normal pulmonary function tests and no history of smoking (Table 2). Informed consent was obtained from each patient and normal volunteer under a protocol approved by the institutional review board.

### Cell preparation and culture

Peripheral blood obtained from patients with active sarcoidosis and healthy donors was anticoagulated with heparin, and PBMCs were separated on a Ficoll-Hypaque (Pharmacia Biotech AB, Sweden) density gradient. To assess the production of sHLAs by cells, PBMCs were cultured in 1 ml of 2×10⁶ endotoxin-tested RPMI 1640 (complete RPMI, Gibco BRL, Paisley, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, HEPES buffer and 10% heat-inactivated (56°C for 1 hour) FBS (ICN Flow, Costa Mesa, CA, USA), and incubated with either lipopolysaccharide (LPS from *Escherichia coli*, serotype 0127: B8, Sigma Chemical Co., St. Louis, MO, USA) or phytohemagglutinin (PHA, Sigma Chemical Co.). After cells were incubated at 37°C in a water-jacketed 5% CO₂ incubator for 24 to 72 hours, the cell free supernatant fluid was collected for determination of sHLAs concentrations.

BAL was performed using 150 ml of normal saline solution injected via a fiberoptic bronchoscope in 50 ml aliquots, with immediate vacuum aspiration after each aliquot. The fluid was filtered through sterile gauze and its volume measured. Lung cells were separated from BALF by centrifugation, washed three times in PBS, and then counted. Cell differential counts were carried out applying morphologic criteria to cytocentrifuged smears stained with Wright-Giemsa. Cell pellets were resuspended in complete RPMI 1640 containing 10% FCS and allowed to adhere to plastic culture dishes at 37°C in a water-jacketed 5% CO₂ incubator. After 1 hour incubation, the cells were washed vigorously to remove non-adherent cells. Adherent cells were then removed from dishes by gentle scraping.

### Table 1. Subjects

<table>
<thead>
<tr>
<th></th>
<th>Sarcoidosis patients</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>94</td>
<td>32</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>57/37</td>
<td>13/19</td>
</tr>
<tr>
<td>Age, yr</td>
<td>51.4±15.4*</td>
<td>50.6±15.3</td>
</tr>
</tbody>
</table>

*Data given are mean±SD value.

### Table 2. Pulmonary Function Test and Bronchoalveolar Lavage Analysis

<table>
<thead>
<tr>
<th></th>
<th>Sarcoidosis patients</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary function*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%VC</td>
<td>88.5±15.6</td>
<td>100.2±6.3</td>
</tr>
<tr>
<td>%FEV1.0</td>
<td>80.3±10.3</td>
<td>82.4±9.4</td>
</tr>
<tr>
<td>%DLco</td>
<td>85.4±6.8</td>
<td>96.5±10.5</td>
</tr>
<tr>
<td>Bronchoalveolar lavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent recovery</td>
<td>58±6</td>
<td>59±8</td>
</tr>
<tr>
<td>Total cells/ml (×10⁶)</td>
<td>58±21</td>
<td>50±14</td>
</tr>
<tr>
<td>Differential cell count**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Alveolar macrophages</td>
<td>67±19</td>
<td>88±12</td>
</tr>
<tr>
<td>%Lymphocytes</td>
<td>31±15</td>
<td>11±7</td>
</tr>
<tr>
<td>%Neutrophils</td>
<td>1±1</td>
<td>1±1</td>
</tr>
<tr>
<td>%Eosinophils</td>
<td>1±1</td>
<td>1±1</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>4.7±3.1</td>
<td>0.8±0.7</td>
</tr>
</tbody>
</table>

*Functional data are expressed as percent of predicted normal values. **Differential cell counts are percentages of total cell counts. *p<0.05 compared with normal volunteers.
with a rubber policeman. This procedure increased the proportion of cells morphologically compatible with alveolar macrophages (AMs). Following this multi-step selection procedure, more than 95% of the above cells were viable, as judged by trypan blue exclusion. Cells recovered from BALF (BALF cells) and AMs were resuspended at 5x10^5 cells/ml and after 24 hours of culture in the presence or absence of LPS or PHA, the production of sHLAs was examined.

**Assay for sHLAs**

sHLAs protein levels were measured in samples using an enzyme-linked immunosorbent assay (ELISA) detection kit (Sang Stat Medical Co., Ltd., Menlo Park, CA, USA) according to the manufacturer's recommendations. This assay is a sandwich ELISA for quantitation of soluble HLA antigen (HLA-A, B, C) with the procedure as follows: prepared standard (soluble HLA-B7) or diluted samples were added to wells of the microtiter plates coated with anti-human monomorphic class I monoclonal antibody (clone W6/32) in duplicate, and incubated for 90 minutes at room temperature. The plates were washed 3 times with PBS containing 0.05% Tween 20 (wash solution), followed by addition of horseradish peroxidase-conjugated rabbit anti-human β2-microglobulin polyclonal antibody into each well. After 60 minutes incubation at room temperature, the plates were washed 3 times with wash solution and finally, o-phenylene-diamine substrate solution was added and the plates were incubated at room temperature for 20 minutes in the dark. The color reaction was stopped with 1N HCl and the absorbance of each well was determined at 490 nm. A standard curve was prepared from four sHLAs standards. The minimum value for detection was 3 ng/ml. A preliminary study showed serum levels of sHLAs in patients to be higher than in control subjects. Thus, the sera from patients were diluted 1:50 with specimen diluents, while those from controls were diluted 1:25. Culture supernatants were diluted 1/2. BALF were concentrated 20 fold by MINICON-B (Amicon Division, W.R.Grace&Conn Co., Ltd., Beverly, MA, USA).

**Serum angiotensin-converting enzyme (ACE) level measurement**

Serum ACE levels were measured by a colorimetric method (Colorimetric assay kit, Fujizoki, Assay, Tokyo) using p-hydroxyhippuryl-L-leucine as the substrate (16).

**Statistical analysis**

Comparison of group data was accomplished using the unpaired two-tailed t test for data that were normally distributed. Data that were not normally distributed were compared using the nonparametric Mann-Whitney U test. Linear regression analysis was used to determine the correlation between investigated parameters. In all statistical evaluations, a p value of <0.05 was considered as significant.

**Results**

**sHLAs levels in serum and BALF**

The value for serum sHLAs concentration in the 94 patients with sarcoidosis was 2,501.8±1,921.2 ng/ml (mean±SD), which was significantly higher than that for 32 normal controls (1,230.9±791.6 ng/ml; p<0.05). Patients with active disease showed significantly increased levels of sHLAs as compared with those with inactive disease (3,961.4±1,677.0 and 843.8±715.0 ng/ml, respectively; p<0.001) (Fig. 1). Regarding the chest roentgenographic stage, we observed a tendency for increase in the order I<II<III, but there was no significant difference (data not shown). The results of BALF cells from both groups are summarized in Table 2. The BALF cell count and differential were consistent with active sarcoidosis. As for the concentration in BAL fluid, sHLAs were detectable in eight of 17 patients with sarcoidosis, but it was only detectable in one control. The level was higher in patients with sarcoidosis than in the control (p<0.05, using the nonparametric Mann-Whitney U test, Fig. 2). The concentration did not exhibit any statistical variation with the chest roentgenographic stage and did not correlate with either the percentage of lymphocytes or their CD4+/CD8+ ratio. Because it has been shown that untreated patients with more active clinical sarcoidosis tend to have higher serum ACE levels (12, 17), we next investigated the link with sHLAs in patients. As shown in Fig. 3, there was a significantly positive correlation between serum levels of sHLAs and those of ACE (r=0.46, p<0.05).

**sHLAs levels in supernatants of cultured PBMCs**

To investigate the production of sHLAs by PBMCs, we measured the levels in culture supernatant (Fig. 4). After 24 hours of culture in medium alone, cells from seven of 20 pa-
Figure 2. Soluble HLA class I antigens (sHLAs) level in bronchoalveolar lavage fluid (BALF). Levels of sHLAs in BALF from patients with active sarcoidosis were also higher than in control subjects. The dashed line indicates the lower limit of detection for sHLAs.

Figure 3. Correlation between serum angiotensin-converting enzyme (ACE) and soluble HLA class I antigens (sHLAs) in sarcoidosis cases. A significant positive relation was observed.

Figure 4. Soluble HLA class I antigens (sHLAs) in the supernatant of cultured peripheral blood mononuclear cells (PBMCs) from active sarcoidosis cases (sarcoidosis cases, ○) and control subjects (O). PBMCs (2×10⁶/ml) were cultured for one (24 hours) to three days (72 hours) in the presence (LPS) or absence (Med) of lipopolysaccharide (LPS, 10 μg/ml). After the culture, the supernatants were harvested and assayed for sHLAs by ELISA. The dashed line indicates the lower limit of detection for sHLAs.

Figure 5. Soluble HLA class I antigens (sHLAs) in the supernatant of cultured bronchoalveolar lavage fluid (BALF) cells and alveolar macrophages (AMs) from patients with active sarcoidosis (sarcoidosis cases, □) and control subjects (△). BALF cells (5×10⁶/ml) and AMs (5×10⁷/ml) were cultured for one day with medium alone (Med), medium plus lipopolysaccharide (LPS), or medium plus phytohemagglutinin (PHA, 10 μg/ml). After the culture, the supernatants were harvested to examine the production of sHLAs. Data expressed are mean±SD values.

Patients with active sarcoidosis generated sHLAs. After 72 hours of the same culture condition the number of patients whose PBMCs produced detectable levels of sHLAs was increased (17 of 20), while PBMCs from control subjects produced no amount of sHLAs in either culture condition. On addition of LPS to the medium, PBMCs from patients generated higher amounts of sHLAs compared with those cultured in medium alone. In contrast, those from control subjects only generated limited low amounts, and solely in the presence of LPS for three days.

It has been shown that in vitro stimulation of lymphocytes with a mitogen can induce sHLAs secretion (18), and thus we examined the production of sHLAs by PBMCs upon exposure to PHA. An increase was observed with cells from both active sarcoidosis cases and control subjects, and there was no significant difference between the two groups (125.5±45.4 ng/ml and 108.8±16.7 ng/ml, respectively).
Soluble HLA Class I in Sarcoidosis

Table 3. Sequential Change of Serum Soluble HLA Class I Antigens in Patients with Active Sarcoidosis

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age, yr</th>
<th>Clinical manifestation*</th>
<th>ACE (IU/l) before/after1</th>
<th>SHLA (ng/ml) before/after1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>improvement of muscular lesion</td>
<td>14.4/12.3</td>
<td>4,900/2,400</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>deterioration of muscular lesion</td>
<td>42.5/69.3</td>
<td>3,875/4,800</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>resolution of BHL</td>
<td>36.7/21.8</td>
<td>5,000/3,050</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>progression of lung involvement (from stage I to stage II)</td>
<td>27.4/30.9</td>
<td>1,250/3,050</td>
</tr>
<tr>
<td>5</td>
<td>74</td>
<td>deterioration of muscular lesion</td>
<td>35.9/54.9</td>
<td>4,700/9,000</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>progression of lung involvement (from stage I to stage II)</td>
<td>21.0/26.5</td>
<td>4,950/6,750</td>
</tr>
<tr>
<td>7</td>
<td>54</td>
<td>progression of lung involvement</td>
<td>18.2/23.6</td>
<td>230/1,500</td>
</tr>
</tbody>
</table>

*Change in clinical manifestation is indicated for each sarcoidosis patient. **Serum levels of ACE and SHLA in the period before (before) or after (after) the indicated clinical manifestation. According to the classification system defined by DeRemee (reference 13).

**sHLAs levels in supernatants of cultured BALF cells and AMs**

Regarding sHLAs generation by BALF cells, those from active sarcoidosis cases generated enhanced amounts of sHLAs compared with their counterparts from control subjects. As for the production of these antigens by AMs, the levels of sHLAs in active cases were also elevated. In both groups, however, production of sHLAs from both BALF cells and AMs was not affected by the presence or absence of either LPS or PHA (Fig. 5).

**Sequential change of serum sHLAs in patients with active sarcoidosis**

When 7 patients with active sarcoidosis were sequentially analyzed during the clinical course, sHLAs levels in serum fluctuated in conjunction with both serum levels of ACE and clinical status (Table 3). Increase of sHLAs levels was observed with deterioration of the clinical picture, correlating with changes in ACE.

**Discussion**

We confirmed in the present study that both serum and BALF levels of sHLAs in patients with sarcoidosis are higher than those in control subjects. In sarcoidosis cases, serum concentrations with active disease were significantly higher than with inactive disease, correlating with ACE levels. To our knowledge, this is the first report demonstrating sHLAs in both serum and BALF from patients with sarcoidosis.

It has been documented that certain HLA phenotypes are associated with higher serum concentrations of sHLAs, and individuals with HLA-A23 or HLA-A24 have a higher value than those without these two antigens (19, 20). However, our previous study of the association between HLA antigens and sarcoidosis in Japanese demonstrated the frequencies of these two antigens to be almost the same in both patients and control subjects (21). It is therefore unlikely that a bias of HLA class I alleles in the patients could explain the difference from the controls.

We cannot explain the precise mechanisms of enhanced secretion of sHLAs by both PBMCs and BALF cells from patients with active sarcoidosis. Brieva et al (18) have shown that stimulated PBMCs from normal subjects actively secrete considerable amounts of sHLAs and that lymphocytes, including T cells and B cells, are the main source in their culture condition. They demonstrated that PHA induced a 20-fold mean increase of the secretion, thus it may be possible that using PHA stimulation in our in vitro experiments did not give a precise evaluation for differential production of sHLAs between the two groups. Whereas they also demonstrated that both monocytes and neutrophils produced only background quantities of sHLAs by several stimulations, including LPS, our present data demonstrated that active sarcoid PBMCs produced them in enhanced amounts by stimulation of LPS compared with normal subjects. Some studies speculated that circulating sarcoid PBMCs might be involved in the disease. It has been reported that active sarcoid blood monocytes release increased levels of radical oxidants (22) and that sarcoid peripheral T lymphocytes express functional IL-2 receptors (23). It has also been demonstrated that PBMCs from active patients express relatively low levels of interleukin (IL) 10, which is thought to inhibit activation of cellular immune responses, compared with healthy subjects (24). Thus our present in vitro data regarding the production form PBMCs may reflect an immunologically activated status observed in these cells from patients with active sarcoidosis.

Enhanced production of sHLAs from both BALF cells and AMs in active sarcoid patients could be related also to the immunological activation of the lungs observed in this disease. It has been reported that AMs from active disease sites have several immune reactivities including enhanced IFN-gamma production, inducing immunological features associated with activation of T cells (14, 25–27). It has also been shown that...
CD4⁺CD45RO⁺ T cells at sites of disease proliferate at exaggerated rate and release IL-2 and other cytokines (8–11). Thus further investigations, including immunohistochemical examination of biopsy specimens from patients are needed to clarify the relationship between the activated immunological status of disease sites and the production of sHLAs in sarcoidosis.

The reason why production of sHLAs by both BALF cells and AMs was not affected by in vitro stimulation might be associated with immunological differences of these cells from PBMCs. The lung has the specific immunological status where mucosal tissues are continuously exposed to an abundance of antigens and it has been reported that AMs from healthy subjects are paradoxically ineffective in presenting antigen to T cells (28).

The serum level of sHLAs is high in diseases in which lymphocyte activation plays a pivotal role in disease pathogenesis, and is thought to reflect immune reactivity in host (29). An increase in serum level of sHLAs was found in patients with active SLE (30), HIV infection (5), and chronic hepatitis (6) and monitoring of these antigens is thought to be a useful indicator of activity of these diseases. In other granulomatous lung diseases, it is also reported that active tuberculosis patients were shown to have increased levels of sHLAs in serum (31). We observed that serum levels of sHLAs in patients with sarcoidosis were correlated with those of ACE and fluctuated in conjunction with clinical manifestation. Considering that sarcoidosis is a disease characterized by formation of granulomas, accompanied by systemic immunological features of ongoing activation discussed above, elevation of serum levels of sHLAs may be related to the immune reactivity in this disease and thus measurement of these levels in patients could be useful as a marker of disease activity.

It has been demonstrated that there are at least three forms of sHLAs (32) associated with β2-microglobulin in human. The 44 kDa class I heavy chain is considered to be derived by transmembrane domain is generated by alternative splicing, and membrane shedding, the 39 kDa heavy chain which lacks the membrane domain is thought to represent products from proteolysis of the intact 44 kDa heavy chain. The assay used in this study detects all forms of heavy chains associated with β2-microglobulin, and it is unclear whether any one form of sHLAs is selectively increased in patients with sarcoidosis. In our further studies, it would be of interest to differentially quantitate the serum concentrations of the various forms of sHLAs.

The elevation of serum levels of sHLAs may be related to immune reactivity in the host, while the biological significance and function of sHLAs remains unclear. In regard to the biological significance of sHLAs, particularly after allogenic organ transplantation, Zavazava et al demonstrated that sHLAs inhibit activated T lymphocytes by inducing apoptosis in allospecific fashion (33). The same group has also reported that a high and stable serum concentration of sHLAs of donor origin characterized longterm stable allograft function in cases of liver transplantation (34). Further investigations are needed to clarify the biological significance of sHLAs in the pathogenesis in sarcoidosis.

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