Release of Tumor Necrosis Factor-α From Human Alveolar Macrophages is Decreased in Smokers

Etsuro Yamaguchi, M.D.; Akhide Itoh, M.D.; Ken Furuya, M.D.; Hiroshi Miyamoto, M.D.; Shosaku Abe, M.D.; and Yoshikazu Kawakami, M.D., F.C.C.P.

It is known that smoking affects the development and maintenance of certain types of granulomatous lung diseases. To explore this mechanism(s), we measured tumor necrosis factor (TNF)-α concentrations in the culture supernatants of lipopolysaccharide (LPS)-stimulated alveolar macrophages (AMs) in 13 healthy nonsmokers, 13 healthy smokers, 13 nonsmoking sarcoid patients, and 16 smoking sarcoid patients. We found that the capacity of smokers' AMs to release TNF-α was significantly decreased both in the normal and sarcoid groups. We also confirmed the previous observation that there was an exaggerated TNF release in patients with pulmonary sarcoidosis. These results indicate a significant role of TNF-α in the pathogenetic mechanisms of pulmonary sarcoidosis and suggest the possible involvement of TNF in the mechanisms by which smoking modulates local immune phenomena.

Methods

Study Population

The normal population consisted of 26 healthy subjects; 13 were nonsmokers and 13 were smokers. None had a history of lung disease, and none showed evidence of lung disease by physical examination, chest radiograph, and pulmonary function tests. Their detailed demographic data and BAL findings are shown in Table 1.

The diagnosis of pulmonary sarcoidosis was established in 29 untreated patients; 13 were nonsmokers and 16 were smokers. They had a compatible clinical picture without evidence of mycobacterial, fungal, or parasitic infection, compatible chest radiographic findings, including bilateral hilar and/or mediastinal lymph node enlargement with or without parenchymal infiltrates, and biopsy evidence of noncaseating epithelioid cell granuloma. None had a history of exposure to organic or inorganic materials known to cause granulomatous lung disorders. Their detailed demographic data, chest roentgenographic staging at the time of BAL, and BAL findings are shown in Table 1.

Preparation of BAL Cells

Bronchoalveolar lavage cells (BACs) were obtained by BAL, as described elsewhere. The recovered cells were washed three times with Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY) and suspended in RPMI-1640 (Gibco) supplemented with 10 percent fetal calf serum (FCS) (Gibco) at a concentration of 5 x 10^6 cells per milliliter.

The differential cell count of BAC was obtained from May-Grünewald-Giemsa-stained cytospin preparations.

Isolation of AMs

The BAC suspensions were resuspended once with neuraminidase-

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*From the First Department of Medicine, School of Medicine, Hokkaido University, Sapporo, Japan. This study was supported in part by the Smoking Research Foundation of Japan and by Grants-in-Aid (02404040 and 04454248) for Scientific Research from the Ministry of Education, Science, and Culture of Japan. Manuscript received February 24; revision accepted July 2. Reprint requests: Dr. Yamaguchi, 1st Department of Medicine, Hokkaido University School of Medicine, Kita-15 Nishi-7, Kitaku, Sapporo, Japan 060.
Table 1—Study Subjects and Bronchoalveolar Lavage Characteristics*

<table>
<thead>
<tr>
<th></th>
<th>Normal Subjects</th>
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<th>Sarcoi Patients</th>
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<tbody>
<tr>
<td></td>
<td>Nonsmoker (n = 13)</td>
<td>Smoker (n = 13)</td>
<td>Nonsmoker (n = 13)</td>
<td>Smoker (n = 16)</td>
</tr>
<tr>
<td>Male/female</td>
<td>67/12</td>
<td>36±3</td>
<td>4/9</td>
<td>10/6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>42±14</td>
<td>43±15</td>
<td>36±15</td>
<td>33±13</td>
</tr>
<tr>
<td>Stage, O/I/II/III</td>
<td>–</td>
<td>–</td>
<td>3/5/4/1</td>
<td>3/5/0</td>
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<tr>
<td>Smoking index, pack-year</td>
<td>0</td>
<td>29±23</td>
<td>0</td>
<td>16±18</td>
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<tr>
<td>Bronchoalveolar lavage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells, x 10^6/ml BALF</td>
<td>10±4</td>
<td>39±31</td>
<td>20±10</td>
<td>31±16</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>87±2</td>
<td>96±3</td>
<td>60±2</td>
<td>85±13</td>
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<tr>
<td>Lymphocytes, %</td>
<td>12±8</td>
<td>3±2</td>
<td>40±33</td>
<td>15±13</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>0.5±0.6</td>
<td>0.3±0.4</td>
<td>0.3±0.5</td>
<td>0.3±0.4</td>
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<tr>
<td>Eosinophils, %</td>
<td>0.1±0.1</td>
<td>0.4±0.7</td>
<td>0.1±0.1</td>
<td>0.2±0.3</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SD.

TNF-α Release by AMs

The purified AMs were suspended in "complete medium" (RPMI-1640 containing 10 mmol/L HEPES, 5 x 10^-5 mol/L 2-mercaptoethanol, 100 U/ml penicillin, and 100 mg/L streptomycin) supplemented with 5 percent heat-inactivated FCS and 20 mg/ml of lipopolysaccharide (LPS) (Escherichia coli, 0127:B8; Difco, Detroit, Mich) at a concentration of 1 x 10^6 cells per milliliter. The cell suspension was plated in 96-well, flat-bottomed, tissue culture plates (Falcon 3072; Becton Dickinson, Oxnard, Calif) in 200-μl aliquots and incubated in a humidified atmosphere of 5 percent CO_2 in air at 37°C for 48 h. The culture supernatants were collected by centrifugation at 3,000 rpm for 10 min, sterilized by filtration, and stored at -20°C until assayed.

Assay of TNF-α

TNF-α concentrations in the cell-free supernatants of AMs were determined by a TNF-α-specific, solid-phase enzyme-linked immunosorbent assay (ELISA) system. Ninety-six-well immunoassay plates (Falcon 3915, Becton Dickinson, Lincoln Park, NJ) were coated overnight at 4°C with monoclonal antibody (II 7C2, provided by Asahi Chemical Industry Co, Ltd, Shizuka, Japan) directed against recombinant human TNF-α. After four washes with 0.2 percent octoyxol-9 (Triton X-100) in phosphate-buffered saline solution (PBS), 200-μl aliquots of test samples and recombinant human TNF-α references prediluted with 0.1 percent gelatin in PBS were added to each well. The plates were incubated overnight at 4°C and washed again four times with the washing buffer. The second monoclonal antibody (IV 3E5, provided by Asahi Chemical Industry Co, Ltd) conjugated with peroxidase was then added to each well. The plates were incubated for 6 h at room temperature and washed in the same manner. One hundred fifty microliters of a substrate solution (30 mg of o-phenylenediamine and 7 μl of hydrogen peroxide solution in 20 ml of 0.1 mol/L citrate-phosphate buffer, at a pH of 5.0) was added to each well. After incubation at room temperature for 15 min, the reaction was stopped by adding 50 μl of 4.5 mol/L H_2SO_4 solution. The reaction product was measured at 492 nm with an automated microplate reader. The sensitivity of this assay method was 0.5 U/ml. The two monoclonal antibodies used to capture human TNF-α did not react with human TNF-β or TNF-α of other species.

Comparisons between smokers and nonsmokers or between normal subjects and patients with pulmonary sarcoidosis were carried out using the unpaired Student's t test. For all comparison, a p value of less than 0.05 was considered to be significant.

RESULTS

We first examined the time course of TNF-α release by AMs at the LPS concentration of 20 μg/ml in four patients with pulmonary sarcoidosis (two nonsmokers, two smokers). The AMs were cultured for 12, 24, 48,
Patients were cytotoxicity viability for T-bet, Sarcoid smaller in the release, significantly increasing patients subjects. Accordingly, then and supernatants and of FcγRIIu 200 250 100 300 50 100 150 200 250 300 0 1.25 5 20 80 LPS (μg/ml) TNF-α (U/ml) Figure 2. TNF-α release from alveolar macrophages in the presence of increasing concentrations of LPS (n = 4). Data are presented as mean ± SE.

and 72 h, and TNF-α concentrations in the culture supernatants were determined by ELISA. As shown in Figure 1, the release of TNF-α increased over time, and plateau levels were reached by 24 h. The levels then remained stable during the following 24 h. Accordingly, a 48-h time point was chosen for assessing TNF-α release from AMs.

We next examined the optimal concentration of LPS for the release of TNF-α. The AMs from two normal subjects (one nonsmoker, one smoker) and two sarcoid patients (one nonsmoker, one smoker) were incubated with various concentrations of LPS for 48 h (Fig 2). Increasing concentrations of LPS led to a dose-dependent increase of TNF release. However, the viability of AMs cultured at 80 mg/L of LPS was significantly decreased in nonsmokers compared to that at 0 mg/L (data not shown). Weighing the potential cytoxicity on AMs and enhancing effect on TNF release, a concentration of 20 mg/L was chosen for the following study.

The AMs from normal subjects and sarcoid patients were incubated for 48 h in the presence of 20 mg/L of LPS, and the concentrations of TNF-α in the culture supernatants were quantitated by ELISA. As shown in Figure 3, smokers' AMs released significantly smaller amounts of TNF-α both in the normal and sarcoid groups (normal smokers vs normal nonsmokers, 187 ± 143 SD vs 328 ± 136 U/ml; smoking sarcoid patients vs nonsmoking sarcoid patients, 322 ± 160 SD vs 570 ± 329 U/ml). Meanwhile, in both smokers and nonsmokers, the levels of TNF-α were significantly increased in sarcoid patients. There was a weak but significant positive relationship between the proportion of BAL lymphocytes and the levels of TNF-α (r = 0.366, p<0.01, data not shown) when all study subjects were combined.

**DISCUSSION**

We made two important observations in this study. We first confirmed previous observations that AMs from sarcoid patients have an increased capacity to release TNF-α. Tumor necrosis factor was first described in 1975 as a necrosing factor for solid tumors found in the sera of BCG-immunized mice challenged with LPS. Subsequent investigations have shown that the biologic activities of this cytokine extend well beyond the realm of antitumor biology. In the respiratory system, TNF is an important mediator in the pathogenesis of a variety of inflammatory processes. In a murine model of silicosis, a single instillation of silica into lungs leads to a marked increase in the level of lung TNF-α mRNA. Elevated levels of plasma TNF-α have been reported in patients with cystic fibrosis. TNF-α is also believed to play a substantial...
role in pulmonary injury during sepsis.10
There is, in addition, increasing evidence in support of the theory that TNF may play an important role in granulomatous inflammation. For example, immune-reactivity to TNF-α is frequently observed in sections of granulomatous lymphadenitis, and its staining is confined to the epithelioid macrophages forming the granuloma.20 In vitro granulomas can be formed by culturing murine spleen cells in the presence of agarose beads coupled to recombinant TNF-α.21
In pulmonary sarcoidosis, Bachwitz and his colleagues7 were the first to report increased TNF production by LPS-stimulated AMs. Their observation has been confirmed by other investigators.6,27 In the present study, we also found a significantly increased release of the cytokine from LPS-stimulated AMs in both smoking and nonsmoking sarcoid patients compared to normal counterparts. Thus, it now appears to be established that TNF is involved in the pathogenetic mechanism of sarcoid lesions.
We noted, secondly and most importantly, that LPS-stimulated AMs from smokers release less TNF-α than do those from nonsmokers, both in normal subjects and in patients with pulmonary sarcoidosis. Tumor necrosis factor can facilitate the capacity of T cells to proliferate in response to mitogens or antigens.22 It can also enhance the adhesiveness of T cells to endothelial cells by generating a binding receptor.23 Another possible role of TNF in promoting granuloma formation may be mediated by stimulating AMs and vascular endothelial cells to produce IL-1,34,35 which has been reported to be another key monokine in the formation of in vitro granulomas.21 Furthermore, TNF and IL-1 can serve as a stimulus for the secretion of the novel monocyte-specific cytokine, monocyte chemotactic protein-1 (MCP-1) by type-II-like pulmonary epithelial cells.26 Because of the close proximity of AMs and epithelial cells, networking between immune and nonimmune cells is most likely to be operative in pulmonary sarcoidosis. In this connection, pulmonary fibroblasts and epithelial cells share similar signal specificity in their production of IL-8, as they respond to TNF and IL-1.27,36 IL-8 has been shown to be chemotactic to T cells as well as neutrophils,29 and increased release of IL-8 has been demonstrated in culture supernatants of BAC in active pulmonary sarcoidosis.30 Since the accumulation of AMs and lymphocytes in the lung represents a hallmark of active pulmonary sarcoidosis, the decreased capacity of smokers' AMs to release TNF may provide partial protection against the development and/or maintenance of the disease.
We have previously demonstrated that the release of IL-1 β by AMs on in vitro stimulation is also reduced in smokers.12 IL-1 and TNF are two structurally unrelated monokines, and certain specific activities are primarily mediated by either TNF or IL-1. However, they share numerous biologic properties. Although the regulation of gene expression of these cytokines is controlled by different mechanisms,31 there was a positive relationship between the concentrations of IL-1 β and those of TNF-α in the culture supernatants of AMs in the current study (our unpublished observation). In addition, it has been shown that TNF induces IL-1 release by AMs in experimental animals,34 thus forming a potentially powerful amplification loop that may contribute to a prompt immune response. In this context, inflammatory and immune processes in the lungs of smokers could be markedly altered by relative lack of these two cytokines. Since they are involved not only in hypersensitivity immune processes but also in the host defense mechanisms against infectious agents and neoplasms,32,33 the decreased release of these cytokines may lead to the increased risk of cigarette smokers having a variety of lung disorders develop.
The precise cellular mechanism of down-regulation of monokine production by smoking is currently unknown. However, it should be emphasized that our observation can be viewed as lowered responsiveness of AMs to LPS in smokers. This may be a reflection of reduced priming effects on smokers' AMs by the other locally produced cytokines whose production may also be affected by smoking. Granulocyte-macrophage colony-stimulating factor is a candidate mediator because it can induce the production of TNF-α by macrophages in response to interferon-γ and LPS34 and is present in the sarcoid lung.4 Another possible mechanism may be related to LPS receptor of AMs. Current concept of signal transduction mechanism of LPS is that LPS first interacts with LPS-binding protein (LBP) in serum, and LPS/LBP complexes then induce cellular responses by binding to their receptor CD14.35 Accordingly, future studies should be directed toward investigating the differences in the cell surface expression of CD14 antigen and changes in signal transduction efficiency via this molecule between smokers and nonsmokers.
Finally, our observation in the present study also indicates the need for special consideration of smoking status when we attempt to evaluate the release of cytokines from human AMs.

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