Smoking and Interleukin-1 Activity Released from Human Alveolar Macrophages in Healthy Subjects*

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To evaluate the activation of alveolar macrophages from smoking, we studied interleukin-1 (IL-1) activity released from alveolar macrophages in eight healthy smokers, compared to 12 healthy nonsmokers. We used 24-hour culture supernatants containing IL-1 of bronchoalveolar lavage fluid (BALF) macrophages/blood monocytes with or without LPS stimulation. Using C3H/HeJ thymocyte PHA costimulation assay, we found that IL-1 activity released from LPS-stimulated BALF macrophages was significantly higher in smokers (2.39 ± 0.33 U/ml) than in nonsmokers (1.47 ± 0.19 U/ml, p < 0.05). We also detected IL-1 inhibitory activity in supernatants by using IL-1 inhibitory assay. The inhibitory activity was higher in nonsmokers than in smokers especially under LPS stimulation. The presence of inhibitory factors other than prostaglandin-E$_2$ was suggested from the differential response to the addition of indomethacin into cultures from nonstimulated and LPS-stimulated supernatants of BALF macrophages. (Chest 1988; 94:694-700)

Smoking plays a significant role in the pathogenesis of pulmonary emphysema, chronic airflow limitation, fibrotic lung diseases, and lung cancer. In addition to a direct injurious effect, tobacco smoke also stimulates immune and inflammatory cells. The continuous stimulation by smoking results in the activation of alveolar macrophages, which then release various enzymes, mediators and oxidants, all of which cause injury to the lung.

Interleukin-1 (IL-1) is an inflammatory monokine with multiple effects on many target cells, causing T cell activation, B cell activation and fibroblast activation.

It has been reported that healthy smokers have a significantly increased number of macrophages in bronchoalveolar lavage fluid (BALF)* but there are no reports about whether IL-1 activity released from smokers' macrophages is higher than that released from nonsmokers.

We studied IL-1 activity and its regulation by BALF macrophages in healthy subjects. We detected differences in IL-1 secretory activities and IL-1 inhibitory activities between smokers and nonsmokers. The presence of IL-1 inhibitory factors other than prostaglandin-E$_2$ (PG-E$_2$) was suggested from the differential response to the addition of indomethacin (IM) into cultures of nonstimulated and LPS-stimulated supernatants of BALF macrophages.

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Materials and Methods

Study Population

We studied 20 healthy volunteers; 12 were nonsmokers and eight smokers. The smokers smoked 24.4 ± 3.4 of cigarette per day, with Brinkman index of 333 ± 124 pack years (mean ± SE). None of the subjects had any signs and symptoms of infection or inflammation. X-ray films, pulmonary function, and routine laboratory test results were normal.

Preparation of BALF and Blood Mononuclear Cells

The BAL was performed as previously described with slight modification. Briefly, the upper airways were anesthetized with 4 percent xylcaine. The fiberoptic bronchoscope was inserted into the tracheobronchial tree and wedged in the right middle lobe. A lavage was performed using warm (37°C) saline solution (50 ml × 6 times). Recovered BALF cells were filtered and centrifuged for 1,200 rpm ten minutes at 4°C twice to prepare BALF cell fractions (5 × 10$^6$/ml) in Eagle's medium solution. Cell populations in BALF were counted in cytocentrifuged specimens after May-Grünwald-Giemsa staining.

Blood mononuclear cells, obtained from heparinized blood by Ficoll-Hypaque centrifugation, were suspended in Eagle's MEM medium solution at a concentration of 5 × 10$^6$/ml.

Purification of BALF and Blood T Cells

The BALF and blood mononuclear cell fractions were purified by rosetting them with neuraminidase-treated sheep red blood cells at 4°C into BALF and blood T cell fractions containing more than 80 percent of T cells. The OKT4$^+$ and OKT8$^+$ cells in the T cell fractions were identified by using anti-OKT4 and anti-OKT8 monoclonal antibody with a cytoflowmeter.

Purification of BALF Macrophages and Blood Monocytes

Nonrosette forming cell fractions (E$^+$ cells) were further purified by plastic adhesion at 37°C, 60 minutes. Nonrosette forming adherent cells were more than 95 percent nonspecific esterase positive and will be referred to as the monocyte/macrophage fraction.
Preparation of Culture Supernatants Containing IL-1 Activity

The BALF macrophages and blood monocytes were washed, resuspended in RPMI-1640 medium with 15 percent fetal calf serum (FCS), and placed into 24-well tissue culture plates at a density of 1 x 10^5/ml, with and without various amounts of lipopolysaccharide B (LPS B E coli 055: B5). In some experiments, we added IM (5 x 10^-9 M). Cultures were incubated for 24 hours at 37°C in 5 percent CO_2 atmosphere. Supernatants were then harvested and stored at -70°C for later use.

**IL-1 Assay (Assessment of Supernatants for IL-1)**

The IL-1 activity was measured by a standard murine thymocyte proliferation assay. Thymocytes were prepared from four to six-week old C3H/HeJ mice and cultured at 1 x 10^5 cells/ml. An IL-1 standard and the experimental IL-1 supernatants were diluted serially in 10 percent FCS-RPMI supplemented with antibiotics. Aliquots of supernatants were added to thymocytes with triplicate wells set for each variable. The thymocytes were cultured with 5 μg/ml of PHA-P HA16 at 37°C in 5 percent CO_2 for three days. [H]-thymidine (0.5μCi) was added 12 hours prior to termination of cell culture. The culture was harvested onto filter-paper discs and the [H]-thymidine uptake was assayed by liquid scintillation spectrophotometry.

The IL-1 activity in the supernatants was defined as showing the duplicated uptake of [H]-thymidine into thymocytes in the presence of PHA alone as 1.0 U/ml.

**IL-1 Inhibitory Assay**

We examined the serial suppression of supernatants to thymocyte proliferation in the response to standard human IL-1. The IL-1 inhibitory activity (percent) was expressed as follows:

\[
\frac{1 - \text{standard IL-1 (cpm)} \text{+ supernatants (cpm)}}{\text{standard IL-1 (cpm)}} \times 100
\]

**Effect of Purified PG-E_2 on IL-1 Activity Released from Monocytes**

We evaluated the effect of PG-E_2 as one inhibitory factor on IL-1 activity, released from blood monocytes which did not show a significant presence of IL-1 inhibitory activity. Murine thymocytes were cultured in the presence of the supernatants (100-fold dilution) plus various amount of purified PG-E_2.

**Statistical Evaluation**

All results were expressed as mean ± SE. Statistical comparisons were made by using Student's two tailed t-test.

**RESULTS**

**BALF Cell Findings**

In smokers, there was a significant (p<0.05) increase in recovered cells per milliliter and macrophages (percent). The lymphocyte count and CD4+/CD8+ ratio were significantly (p<0.05) reduced in smokers as compared to nonsmokers. The percentages of neutrophils and eosinophil were similar in both groups (Table 1).

**IL-1 Activity Released from BALF Macrophages and Blood Monocytes**

Spontaneously released IL-1 activity was much lower in BALF macrophages than in blood monocytes. When BALF macrophages and blood monocytes were stimulated with LPS, the IL-1 activity considerably increased compared to nonstimulated conditions. The IL-1 activity in supernatants from nonstimulated cells was similar in nonsmokers and smokers. The IL-1 activity released from LPS-stimulated BALF macrophages was significantly higher in smokers than in nonsmokers (p<0.05) (Table 2). The LPS stimulation of blood monocytes showed similar increase in IL-1 secretion.

**IL-1 Activity and the Concentration of LPS**

It was not possible to demonstrate a typical dose-relationship between the range of LPS used (0.01 to 100 μg/ml), and IL-1 activity in the supernatants released from BALF macrophages and blood monocytes. The finding that IL-1 activity in smokers was significantly (p<0.01) higher than those in nonsmokers was found at the concentration of LPS of 0.01-100 μg/ml, both in BALF macrophages and blood monocytes (Fig 1).

**Table 1—Findings of BALF Cells and Blood Cells in Healthy Individuals**

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
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<tbody>
<tr>
<td>Number (M,F)</td>
<td>12 (7.5)</td>
<td>8 (8.0)</td>
</tr>
<tr>
<td>Age</td>
<td>39.5±3.5*</td>
<td>31.5±4.9</td>
</tr>
<tr>
<td>BALF cells (1.0 x 10^5/ml)</td>
<td>0.71±0.15</td>
<td>1.57±0.36†</td>
</tr>
<tr>
<td>BALF macrophage (%)</td>
<td>87.8±1.76</td>
<td>95.0±0.82†</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>10.9±1.76</td>
<td>4.4±0.70†</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>1.12±0.48</td>
<td>0.8±0.29</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>0.33±0.06</td>
<td>1.6±0.87</td>
</tr>
<tr>
<td>BALF T cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+/CD8+ ratio</td>
<td>2.43±0.31</td>
<td>0.95±0.07†</td>
</tr>
<tr>
<td>Blood T cell</td>
<td></td>
<td></td>
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<tr>
<td>CD4+/CD8+ ratio</td>
<td>1.73±0.21</td>
<td>1.77±0.23</td>
</tr>
</tbody>
</table>

*Mean±SE.
†Significant to nonsmokers (p<0.05).

**Table 2—IL-1 Activity in Culture Supernatants (100-Folds Dilution) With and Without LPS Stimulation**

<table>
<thead>
<tr>
<th></th>
<th>BALF Macrophage</th>
<th>Blood Monocyte</th>
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<tbody>
<tr>
<td></td>
<td>Nonstim</td>
<td>LPS-stim</td>
</tr>
<tr>
<td>Nonsmoker (n = 12)</td>
<td>0.73±0.11*</td>
<td>1.47±0.19†</td>
</tr>
<tr>
<td>Smoker (n = 8)</td>
<td>0.80±0.13</td>
<td>2.39±0.33†</td>
</tr>
</tbody>
</table>

*Mean±SE.
†Significant to nonsmoker (p<0.05).
‡Significant to nonstimulated BALF macrophage (p<0.05). We used 1.0 μg/ml as the concentration of LPS.
Inhibitory Detection of IL-1 activity by IL-1 Inhibitory Assay

The IL-1 inhibitory activity was evaluated at the 10-, 100-, and 1,000-fold dilution of supernatants from BALF macrophages with and without LPS stimulation using IL-1 inhibitory assay.

In nonsmokers, IL-1 inhibitory activity at tenfold and 1000-fold dilution was significantly higher in LPS stimulated than in nonstimulated macrophages. But in the group of smokers, the above findings tended to be reversed. At tenfold dilution, significant inhibitory activity was detected in nonsmokers samples, independent of LPS stimulation, but among smokers, LPS stimulation did not enhance the IL-1 inhibitory activity (Fig 4). There was a significant difference in IL-1 inhibitory activity between nonstimulated and LPS stimulated macrophage culture supernatants in smokers.

Effect of IM on IL-1 Activity Released from BALF Macrophages and Blood Monocytes

The effect of IM on the release of IL-1 inhibitory factors from BALF macrophages was examined in five nonsmokers, and from blood monocytes in seven nonsmokers (Fig 5). The effect of IM was expressed as the percentage of IL-1 activity in the presence of IM to that in the absence of IM.

The addition of IM produced significant enhancement in the LPS stimulated BALF macrophages, but no change was found in the nonstimulated BALF macrophages. Slight enhancement was found in LPS-stimulated blood monocytes, but no change in those nonstimulated.

Suppressive Effect of Purified PG-E2 on IL-1 Activity Released From Blood Monocytes

The effect of PG-E2 as an inhibitory factor on IL-1 activity from blood monocytes of three nonsmokers was evaluated. No evidence of IL-1 inhibitory activity was found in these samples. The addition of PG-E2...
into cultures produced suppression of IL-1 activity which while not significant was present at every dose of PG-E₂ (0.5 to 500 ng/ml) (Fig 6).

**DISCUSSION**

The role of IL-1 in the inflammatory process in the lung has been widely discussed. Some have referred to the influence of smoking on the inflammatory process and lung injury in pulmonary emphysema and fibrotic lung diseases. Lavage cell populations from healthy smokers display characteristic changes. Smokers' BALF contains an increased number of alveolar macrophages which are also morphologically different from the nonsmokers. The BALF macrophage in healthy smokers is about three-times more active metabolically and includes various enzymes. Collectively, these features suggest that smokers' alveolar macrophages are activated in vivo.

Earlier reports indicated that the accessory functions, particularly those associated with antigen presentation and activation of T lymphocytes of alveolar macrophage in smokers, were impaired. Other reports have failed to reproduce these results. Using PHA, anti-CD3 antibody as mitogens, we have observed a significant enhancement of the accessory function of alveolar macrophage as related to T cells in healthy smokers (unpublished data). The relationship between the accessory function and IL-1 activity of BALF macrophages has not been completely investigated. The effector functions of smokers' alveolar macrophages are altered. There is an enhanced production of cytotoxic effector molecules (such as superoxide anion) after stimulation and reduction in regulator molecules derived from arachidonate cascade. Smokers' alveolar macrophages are a source of chronic tissue damage. Therefore, it is important to examine IL-1 activity released from BALF macrophages and its regulation by IL-1 inhibitory factors in chronically inflamed lungs, such as those of smokers.

Our study population showed the typical increase of BALF macrophages in smokers. These macrophages released significant amounts of IL-1 after LPS stimulation. This feature, also observed in blood monocytes, raises the possibility of a flow of stimulating substances from the lung to systemic circulation (Table 2).

An earlier report commented that alveolar macrophages were at least 1,000 times less sensitive to LPS than blood monocytes, and this difference was due neither to the release of substances by macrophages, which inhibited lymphocyte proliferation in response to IL-1, nor to degradation of IL-1 by macrophages.

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**FIGURE 3.** Dilution curves of culture supernatants. Culture supernatants were diluted by culture medium and IL-1 activity of diluted supernatants were assayed (12 cases of nonsmokers, eight cases of smokers). Dotted lines express the supernatants without stimulation and solid lines express those with LPS stimulation. Open circles and closed squares express smokers. Each point shows mean ± SE.
Our data failed to show a significant difference between BALF macrophage and blood monocyte IL-1 activity after LPS stimulation both in nonsmokers and smokers. However, in nonstimulated samples, a significant increase in IL-1 activity was observed in monocytes, as compared to BALF macrophages (Table 2). This observation is contrary to previous reports in which no IL-1 activity was detected in nonstimulated samples. As to such a discrepancy, we excluded the following possibilities. First, we routinely tested for LPS contamination with the Limulus gelatin test prior to LPS stimulation and the results were negative. Second, we examined the copresence of IL-2 which could stimulate murine thymocyte proliferation but could not detect a significant activity. Third, we examined IL-1 activity of some supernatants of blood monocytes using IL-1β-radioimmunoassay. When compared with the murine thymocyte proliferation assay, IL-1 activity by nonstimulated monocyte supernatants was low but detectable (4.2±1.1 ng/ml in nonsmokers, 5.0±1.4 ng/ml in smokers). This IL-1 activity detected in nonstimulated cultures may reflect the activation of monocytes/macrophones in vitro during a plastic adhesion and following 24 hours of culture even in the absence of LPS. Our findings are compatible with the previous report of Treves et al which showed a constitutional secretion of IL-1 activity but was related to the maturational state of the mononuclear phagocytes.

![Figure 4](image-url)  
**Figure 4.** IL-1 inhibitory activities released from supernatants of BALF macrophage with or without LPS stimulation by using IL-1 inhibitory assay. IL-1 inhibitory activity was evaluated by the serial suppression of supernatants to thymocyte proliferation in the response to standard human IL-1. *Dotted lines* express supernatants without stimulation and *solid lines* express supernatants with LPS stimulation. *Open circles* and *open squares* express nonsmokers and *closed circles* and *closed squares* express smokers. Each point shows mean ± SE. IL-1 inhibitory activity was expressed as follows:

\[
\text{IL-1 inhibitory activity (\%)} = \left(1 - \frac{\text{standard IL-1(\Delta cpm)} + \text{sup.(\Delta cpm)}}{\text{standard IL-1 (\Delta cpm)}}\right) \times 100
\]

![Figure 5](image-url)  
**Figure 5.** The effect of indomethacin (IM) on IL-1 activity released from BALF macrophage and blood monocyte. Cultures were incubated for 24 hours at 37°C in a 5 percent CO₂ atmosphere with or without addition of IM (5 x 10⁻⁷M) into culture plate at the initiation of culture. Supernatants (seven cases of blood monocytes, five cases of BALF macrophages) were harvested and assayed using murine thymocyte proliferation assay. The effect of IM was expressed as the percentage of IL-1 activity in the presence of IM to those in the absence of IM. *Open circles* express IL-1 activity of nonstimulated samples and *open squares* express those of LPS stimulated ones. Each point shows mean ± SE.
from normal human monocytes.

The dose-response study revealed no difference of sensitivity to LPS between monocytes and macrophages from smokers as well as nonsmokers.

It has recently been demonstrated that human alveolar macrophages release not only IL-1, but also inhibitors of IL-1.16,17 Endotoxin is an effective stimulus for the release of both factors. The thymocyte proliferation assay simply measures the net effect of these positive (IL-1) and negative (IL-1 inhibitors) factors in the supernatants of the alveolar macrophages. We detected IL-1 inhibitory factors in BALF macrophage supernatants with or without LPS stimulation and in LPS stimulated monocyte supernatants, from the dilution curve of supernatants (Fig 2 and 3). The deviation from the standard IL-1 dilution curve is shown at tenfold dilution. We noted that this decrease was not due to any cytotoxic factor in the supernatants at tenfold dilution, by performing a trypan blue dye exclusion test. The inhibitory factors could be directly detected by IL-1 inhibitory assay (Fig 4), in which significant decrease of inhibitory activity occurred in smokers at a tenfold dilution of samples. This inhibitory activity significantly decreased after LPS stimulation in smokers’ macrophages. It is likely that BALF macrophages release a variety of inhibitors of IL-1 after stimulation with LPS. One of these inhibitors is PG-E₂,20,21 but the other inhibitors are less well defined.22-24 A previous report showed that LPS stimulated alveolar macrophages released large amounts of PG-E₂ at 24 hours.20 In our experiment, the addition of IM into cultures enhanced IL-1 activity in LPS stimulated BALF macrophage supernatants, but not in nonstimulated BALF macrophages, and not in blood monocytes (Fig 5). Therefore, it is likely that PG-E₂ is produced after LPS stimulation, and different IL-1 inhibitory factors may be produced from nonstimulated macrophages.

The suppressive effect of PG-E₂ was confirmed. We selected the nonsmokers’ blood monocytes because of low levels of IL-1 inhibitory activity, and IL-1 released from nonsmokers’ monocytes decreased in the presence of purified PG-E₂ in culture (Fig 6). This finding was shown both in nonstimulated and LPS stimulated monocyte cultures. As for other IL-1 inhibitory factors, inter-a-trypsin inhibitor,25 inhibitor of plasminogen activator,26 and other proteinous factors22-24 have been demonstrated.

We conclude that cigarette smoking influences the release of IL-1 from LPS stimulated BALF macrophages and blood monocytes. It also apparently causes significant decrease in IL-1 inhibitory activity both in nonstimulated and LPS stimulated BALF macrophages.

The difference in the reactivity of macrophages/monocytes to LPS as a stimulus between nonsmokers and smokers may reflect the presence of a primed state of macrophage/monocyte system brought about by chronic smoking.27 However, the hypothesis of the primed (preactivated) state of BALF macrophage in smokers fails to explain the simultaneous increase in IL-1 activity and significant decrease in IL-1 inhibitory factors.28 It is conceivable that smoking brings BALF macrophages to a primed state that is easy to be stimulated by LPS in the production of IL-1, but to a deactivated state in terms of the production of IL-1 inhibitory factors.27

Though the apparent IL-1 activity in BALF macrophages was lower than that in blood monocytes because of the copresence of IL-1 inhibitory activity,
BALF macrophages released IL-1 in amounts comparable to that released by blood monocytes, independent of smoking.

These findings suggest that BALF macrophages may not have a defect in release of IL-1, even in nonsmokers. The observation that human alveolar macrophages can produce both IL-1 and IL-1 inhibitory factor after stimulation is reasonable, in the context of a regulatory mechanism in the normal human lung. The normal lung suppresses immune responses to a wide variety of antigens to which it is exposed during the normal process of respiration. The release of large amounts of IL-1 inhibitory factors by alveolar macrophages after stimulation may allow these cells to ingest and remove antigens without triggering an immune response to the antigen. From this viewpoint, smoking may disturb the normal regulatory mechanisms in the lung and may cause lung injury.

The differential production/release between IL-1 and IL-1 inhibitory factors in smokers strongly suggests such a process induced by cigarette smoking.

The separation of IL-1 and IL-1 inhibitory activity chromatographically might help to evaluate quantitatively these activities in nonsmokers and smokers.

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