BAL Induces an Increase in Peripheral Blood Neutrophils and Cytokine Levels in Healthy Volunteers and Patients With Pneumonia*

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Study objectives: To examine the peripheral effects of BAL on the neutrophil counts and cytokine levels in the circulation.

Design and methods: WBC counts and plasma cytokines were measured before and 4 h after fiberoptic bronchoscopy (FOB) without further interventions (n=6), or combined with BAL in normal volunteer subjects (n=6), and in patients with bacterial pneumonia (n=4). The bronchus of the right middle lobe was wedged, and three 50-mL aliquots of sterile saline solution was instilled. There was no endotoxin contamination in the saline solution or the fluid obtained through the working channel of bronchoscope.

Results: In volunteers, peripheral WBC counts and the number of nonsegmented and segmented neutrophils increased after the BAL procedure (p<0.05) associated with the increase in plasma concentration (mean±SEM) of interleukin (IL)-6 (0.99±0.32 pg/mL before BAL and 20.38±13.42 pg/mL after BAL; p<0.05) and granulocyte colony-stimulating factor (G-CSF; 14.1±1.7 pg/mL before BAL and 38.5±9.7 pg/mL after BAL; p<0.05). The increase in WBC counts and neutrophil counts was positively correlated to the increase in IL-6 (p<0.05) and the increase in G-CSF (p<0.05). In patients with pneumonia, IL-6 and G-CSF levels were higher after BAL than in normal volunteer subjects (p<0.05). There was no increase in plasma concentration of IL-1β, tumor necrosis factor-α, or IL-8 after BAL in normal volunteer subjects or in patients with pneumonia. FOB without BAL did not increase the WBC count, neutrophil count, or plasma cytokine levels.

Conclusion: The BAL procedure increases the number of WBCs, and segmented and nonsegmented neutrophils in the peripheral circulation as well as circulating IL-6 and G-CSF levels.

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Key words: BAL; granulocyte colony-stimulating factor; interleukin-6; neutrophilia

Abbreviations: ELISA = enzyme-linked immunosorbent assay; FOB = fiberoptic bronchoscopy; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; SIRS = systemic inflammatory response syndrome; TNF-α = tumor necrosis factor-α

BAL is a well-established diagnostic procedure in respiratory and critical-care medicine. Side effects of the procedure are fever, headache, and transient hypoxemia.1,2 Usually, these symptoms begin a few hours after BAL and subside spontaneously within 1 day. It has been reported that cytokines derived from alveolar macrophages, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, induce fever after BAL in the patients with interstitial lung diseases.3 In these patients, the serum concentration of TNF-α was higher before BAL, and the patients with elevated IL-1β concentrations before BAL were more likely to develop fever than were patients with normal cytokine values. This study suggests that the BAL procedure induces a systemic inflammatory response characterized by an increase in circulating cytokine levels.

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A systemic inflammatory reaction has been described in the systemic inflammatory response syndrome (SIRS). The SIRS can be elicited by a wide variety of insults, such as trauma, hemorrhagic shock, and septicemia. A neutrophilia associated with an increase in the percentage of immature (nonsegmented) neutrophils can be seen during SIRS. Macrophages and their products, such as TNF-α, IL-1, and IL-8, and neutrophils have been implicated in the pathogenesis of SIRS. Several studies have suggested that immature neutrophils are less deformable and more likely to sequester in the lung. In these studies, newly released neutrophils have been implicated in mediating endothelial injury in pulmonary microvasculature. These neutrophils may be released from bone marrow by inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, and granulocyte colony-stimulating factor (G-CSF). In humans, studies have shown that BAL causes neutrophil recruitment in the lower bronchial and alveolar spaces, and the signals for these recruitment could be inflammatory cytokines released from alveolar cells such as alveolar macrophages.

We hypothesized that BAL causes a systemic inflammatory response that is characterized by circulating cytokines capable of stimulating the bone marrow to release neutrophils. We postulated that mediators released from the lung contribute to this systemic response. To test these hypotheses, blood cell counts including differentiated WBC counts as well as plasma cytokine levels were measured before and 4 h after fiberoptic bronchoscopy (FOB) with or without BAL in normal healthy volunteer subjects and also in patients with bacterial pneumonia. We expected a systemic inflammatory response 4 h after FOB because increased concentrations of inflammatory cytokines have been reported 4 h after BAL, but not at later times.

Materials and Methods

**BAL**

Twelve healthy volunteers (aged 20 to 24 years) underwent FOB without further interventions (n = 6) or with BAL (n = 6). All were nonsmokers, with no history of bronchial asthma, chronic respiratory tract infection, or a systemic inflammatory disease. They had not received any medication in the past 6 months. All studies were approved by the Human Subject Protection Committee of the Tokyo Dental College, and all subjects gave informed consent to participate in the study. BAL was performed according to American Thoracic Society guidelines. BP, pulse rate, and axillary body temperature were measured before and 4 h after the BAL procedure with the patient in a supine position. Fever was defined as a rise of the body temperature > 37.5°C.

Each subject was premedicated with an IM injection of atropine, 0.5 mg, and hydroxyzine, 25 mg. Local anesthesia of the pharynx and laryngeal area was performed with the inhalation and spray of 4% lidocaine aerosol. Topical anesthesia of the trachea and the bronchial system was obtained by the direct application of 4% lidocaine administered in 1-mL boluses via the working channel of the bronchoscope. The bronchus of the right middle lobe was wedged, and three 50-mL aliquots of a 0.9% sterile saline solution at room temperature was instilled with a syringe through the working channel of the bronchoscope. BAL fluid was recovered by manual aspiration using the attached syringe. The total volume of saline solution instilled into the lung was 150 mL, and 70 to 110 mL of BAL fluid was recovered. The number of cells was counted (model SE-9000; Sysmex; Kobe, Japan). Cytospin with Diff Quick stain (Sigma Chemical; St. Louis, MO) was performed to obtain differential cell counts. Cell differential counts yielded 95.6 ± 0.3% macrophages, 3.1 ± 0.2% lymphocytes, and 1.1 ± 0.1% neutrophils (mean ± SEM). The viability of these cells was assessed with 0.01% trypan blue; > 95% of the cells were viable. In the FOB group, each subject underwent FOB with the same anesthesia and the bronchus of the right middle lobe was wedged, but without further interventions.

**BAL Procedure in Patients With Infectious Pneumonia**

To investigate the effects of the BAL procedure in patients with infection, BAL was carried out in four patients with suspected community-acquired bacterial pneumonia. The patients were from 32 to 64 years old, three were women, and no patients had comorbid conditions such as bronchial asthma, COPD, heart failure, renal failure, or impaired immunity. The BAL in these patients was done to obtain bacterial culture (pneumonia group, n = 4). The chest radiograph of three patients showed consolidation in the right lower lobe, and in the left lower lobe in the other patient. BAL was done in the lobe of consolidation. The BAL procedure was done as described before. The bronchus of the lobe with consolidation imaged with either chest radiograph or chest CT scan was wedged, and 50 mL of 0.9% sterile saline solution at room temperature was instilled with a syringe through the working channel of the bronchoscope. The total volume of saline solution instilled into the lung was 150 mL, and 50 to 100 mL of BAL fluid was recovered. Cell differential counts yielded 27.0 ± 3.3% macrophages, 11.3 ± 4.6% lymphocytes, and 61.8 ± 6.7% neutrophils. The recovered fluid was also used for microbiological investigation.

**Endotoxin Measurement of Instilled Saline Solution**

In order to determine whether fluids instilled through the bronchoscope could be contaminated with endotoxin, both instilled saline solution and the saline aliquots obtained through the working channel of bronchoscope were tested using the limulus amebocytes lysate method (E-Toxate; Sigma Chemical). This is a semiquantitative assay with a sensitivity of 0.1 endotoxin units per milliliter.

**Leukocytes Counts**

Blood samples were obtained from the cubital vein before and 4 h after the bronchoscopy. Blood cell counts were determined on a model SE-9000 (Sysmex), and differential WBC counts were done on Wright's stained blood smears.

**Cytokine Assay**

Plasma concentrations of the cytokines were determined using commercially available enzyme-linked immunosorbent assay
(ELISA) kits. TNF-α, IL-1, and IL-6 were determined by Quantikine high-sensitivity ELISA kits (R&D Systems; Minneapolis, MN). The lower ranges of the test were 0.5 pg/mL for TNF-α, 0.125 pg/mL for IL-1β, and 0.156 pg/mL for IL-6, respectively. IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were determined by Biotrak ELISA kits (Amersham; Buckinghamshire, UK). The lower ranges of the test were 10.0 pg/mL for IL-8 and 2.0 pg/mL for GM-CSF. G-CSF was determined using chemiluminescence enzyme immunoassay method18 (G-CSF CLEIA kit; Chugai Pharmaceutical; Tokyo, Japan), and the lower range of the test was 1.0 pg/mL.

Statistical Analysis

Data are expressed as mean ± SEM, and differences between groups were evaluated by analysis of variance. If differences between groups were significant (p < 0.05), Fisher’s protected least difference test was used as a post hoc test. The values before and 4 h after the BAL were compared using a paired t test. The level of p < 0.05 was accepted as statistically significant.

RESULTS

Endotoxin Assay

There was no endotoxin contamination in the saline solution or the fluid obtained through the working channel of bronchoscope.

Hemodynamics and Fever

There were no differences in BP or heart rate before and after bronchoscopy with or without the BAL procedure, suggesting that there were no differences in cardiac output or pulmonary blood flow. None of the normal volunteer subjects developed fever after bronchoscopy without interventions or with BAL. In patients with bacterial pneumonia, three of four patients developed a fever and tachycardia 4 h after BAL. One of the subjects had fever before the procedure, and two others developed fever after BAL.

Peripheral Blood

Table 1 showed the number of WBC counts, nonsegmented and segmented neutrophils, eosinophils, basophils, lymphocytes, and monocytes. The peripheral WBC counts and the number of nonsegmented and segmented neutrophils increased after the BAL procedure in normal volunteers (p < 0.05) and in the patients with pneumonia (p < 0.05). There was no increase in the number of WBCs or nonsegmented and segmented neutrophils after bronchoscopy without BAL. The number of eosinophils, basophils, monocytes, or lymphocytes did not change after bronchoscopy with or without BAL.

Plasma Cytokines

The plasma cytokine concentrations were shown in Table 2. Baseline values for TNF-α and IL-1β

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before</th>
<th>4 h After Bronchoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOB group</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>WBCs</td>
<td>6,150 ± 548</td>
<td>6,583 ± 694</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3,619 ± 473</td>
<td>4,060 ± 584</td>
</tr>
<tr>
<td>Nonsegmented</td>
<td>42 ± 17</td>
<td>36 ± 12</td>
</tr>
<tr>
<td>Segmented</td>
<td>3,577 ± 481</td>
<td>4,024 ± 584</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>343 ± 90</td>
<td>281 ± 94</td>
</tr>
<tr>
<td>Basophils</td>
<td>28 ± 9</td>
<td>55 ± 15</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1,761 ± 150</td>
<td>1,745 ± 248</td>
</tr>
<tr>
<td>Monocytes</td>
<td>387 ± 125</td>
<td>355 ± 60</td>
</tr>
<tr>
<td>BAL group</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>WBCs</td>
<td>5,200 ± 398</td>
<td>8,533 ± 1,534†</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2,755 ± 250</td>
<td>5,605 ± 1,553†</td>
</tr>
<tr>
<td>Nonsegmented</td>
<td>180 ± 47</td>
<td>338 ± 62†</td>
</tr>
<tr>
<td>Segmented</td>
<td>2,596 ± 230</td>
<td>5,276 ± 1,473†</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>152 ± 58</td>
<td>178 ± 70</td>
</tr>
<tr>
<td>Basophils</td>
<td>42 ± 20</td>
<td>34 ± 24</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1,950 ± 144</td>
<td>2,416 ± 387</td>
</tr>
<tr>
<td>Monocytes</td>
<td>281 ± 51</td>
<td>313 ± 64</td>
</tr>
</tbody>
</table>

Pneumonia group | (n = 4) |                         |
| WBCs | 8,650 ± 1,359 | 11,325 ± 727† |
| Neutrophils | 6,245 ± 1,397 | 9,618 ± 770† |
| Nonsegmented | 67 ± 42 | 633 ± 132† |
| Segmented | 6,179 ± 1,393 | 8,985 ± 787† |
| Eosinophils | 85 ± 24 | 3 ± 3 |
| Basophils | 23 ± 9 | 31 ± 31 |
| Lymphocytes | 1,330 ± 459 | 1,192 ± 191 |
| Monocytes | 805 ± 432 | 481 ± 120 |

*pData are presented as mean ± SEM. †p < 0.05 compared with before BAL.

were similar in all groups and did not increase after bronchoscopy with or without BAL. The plasma concentration of IL-6 before bronchoscopy was higher in the pneumonia group than in the FOB and BAL groups (p < 0.05). The plasma concentration of IL-6 did not change after bronchoscopy without BAL in the normal volunteers (FOB group). The plasma concentration of IL-6 increased after BAL in normal volunteers (BAL group) and in patients with pneumonia (pneumonia group; p < 0.05). The plasma concentration of IL-6 after BAL was higher in the pneumonia group than in the BAL and FOB groups (p < 0.05). The plasma concentrations of IL-8 were below the sensitivity before or after BAL in normal volunteers (<10.0 pg/mL) and did not increase after BAL. The plasma concentration of G-CSF did not differ among the groups before the bronchoscopy, and did not increase after bronchoscopy without BAL in the normal volunteer subjects (FOB group). The BAL increased the plasma concentration of G-CSF in the normal volunteer subjects and in the pneumonia group (p < 0.05). The
The present study showed that saline solution instillation into the normal lung during the BAL procedure increased the number of WBCs and nonsegmented and segmented neutrophils in the peripheral circulation, as well as an increase in the plasma levels of IL-6 and G-CSF. These increases of WBC and neutrophil counts were positively correlated with the increases in the serum concentrations of IL-6 and G-CSF.

Bronchoscopy and BAL induce flu-like symptoms, headache, and fever in up to one third of subjects; fever is more likely when topical anesthetic is instilled through the working channel of the bronchoscope rather than administered by nebulization. Nelson and his colleagues have shown the endotoxin contamination of the lungs during bronchoscopy and suggested that this endotoxin could induce intrapulmonary accumulation of IL-8, IL-1β, and TNF-α. We have used endotoxin-free saline solution to do the BAL, and no subjects developed a fever or a TNF-α response. There was no endotoxin contamination in the samples obtained through the working channel of the bronchoscope. This suggests that endotoxin was not responsible for the systemic inflammation we observed.

The lack of an increase in the plasma cytokine levels after bronchoscopy without BAL suggests that the BAL procedure rather than the bronchoscopy induced the cytokine response, which is supported by studies in children. The mechanisms of the cytokine release by BAL are unclear. The physical process of collecting cells such as the negative pressure with suction could be important in generating the release of cytokines. Alternatively, the changes in surface potency or surface tension induced by the saline solution instillation could stimulate cells. The cell types that released the cytokines include activated monocytes and macrophages, and because macrophages are the predominant cell type in the lung alveoli, it is likely that they are the main source of cytokine production by the BAL procedure.

We showed that the increases of WBC and neutrophil counts were positively correlated with the increases in IL-6 and G-CSF. G-CSF increases the production and release of segmented and nonsegmented neutrophils from the bone marrow and alters neutrophil functions, and we suspect that G-CSF contributed to the neutrophilia after BAL. Endogenous G-CSF serum levels have been shown to be increased as much as 30-fold in patients with severe infection. In the present study, the BAL procedure induced a 2.7-fold increase in the G-CSF concentration in the normal volunteer subjects and a 10-fold increase in patients with pneumonia. The presence of large numbers of inflammatory cells and priming of these cells in the pneumonic process could be responsible for the enhanced cytokine release.
IL-6 is a pleiotropic cytokine involved in the regulation of the immune response, the acute-phase reaction, and hematopoiesis, enhancing IL-3-dependent proliferation of multipotential hematopoietic progenitors in vitro. IV administration of IL-6 induces a biphasic neutrophilia with an initial peak at 1.5 h because of demargination and a second neutrophilia between 4 h and 12 h because of release of marrow neutrophils. IL-6 also induces corticosteroid release, and we have shown that dexamethasone causes a neutrophilia primary by demargination with a minor contribution from bone marrow. Although the increase in the nonsegmented neutrophils signifies a definite bone marrow release response, the high levels of IL-6 induced by the BAL procedure suggest that demargination of intravascular neutrophils also contributed to the neutrophilia we observed. Whether by inducing bone marrow release or by demargination, it is likely that IL-6 significantly contributes to the neutrophilia induced by BAL.

Catecholamines, exercise, and an increase in cardiac output are known to induce an increase in the number of circulating neutrophils by shifting cells from the marginated pool into the circulation. This demargination is not associated with an increase in circulating nonsegmented neutrophils, in contrast to the neutrophilia we observed after BAL. We did not observe any hemodynamic changes during or after the BAL procedure, suggesting that these changes are unlikely to be responsible for the neutrophilia we observed.

Elevated levels of the cytokines, TNF-α, IL-1β, and IL-6, have been suggested to contribute to the systemic inflammatory response after BAL in patients with interstitial lung disease. We have shown that BAL in normal volunteer subjects also induced the release of cytokines into the circulation, and this response was augmented during bacterial pneumonia. We speculate that the BAL procedure in subjects with preexisting lung inflammation could augment the systemic inflammatory response. This could be particularly injurious in subjects with conditions such as septicemia, multiple trauma, shock complicated by the SIRS, and multiple organ failure.

In summary, we have shown that the BAL procedure causes an increase in circulating cytokines, particularly IL-6 and G-CSF. The increase was associated with a systemic inflammatory response characterized by a neutrophilia. This is clearly an important variable for investigation using BAL when studying inflammatory lung conditions. We speculate that the alveolar macrophages make an important contribution to this release of cytokines. Although this inflammatory response induced by BAL was not injurious in the healthy volunteers, it could augment the systemic inflammatory response in subjects with underlying lung diseases.

References


