The usefulness of bronchoalveolar lavage (BAL) fluid cellular analysis in non-human immunodeficiency virus (HIV)-infected patients with *Pneumocystis jirovecii* pneumonia (PCP) has not been adequately evaluated. The objective of this study was to analyze the cellular profiles of BAL fluid and to evaluate their prognostic significance in non-HIV-infected patients with PCP. A 7-year retrospective cohort study of 166 non-HIV-infected adult patients with PCP who underwent BAL was performed in a tertiary care hospital. The median total BAL fluid white blood cell count was 180/µL (interquartile range, 80 to 330) and was unaffected by the severity of PCP. The median percentages of BAL fluid neutrophils, lymphocytes, and alveolar macrophages were 13.1%, 31.7%, and 30.2%, respectively. The median percentage of BAL fluid neutrophils was significantly higher in severe than in mild-to-moderate PCP (20.4% versus 6.0%, *P* < 0.001), as was the absolute neutrophil count (24/µL versus 13/µL, *P* = 0.001). The percentage of BAL fluid neutrophils was an independent predictor of 30-day (adjusted odds ratio [aOR], 1.02; 95% confidence interval [CI], 1.01 to 1.03) and 60-day (aOR, 1.02; 95% CI, 1.01 to 1.04) mortalities. The 30-day and 60-day mortalities increased at rates of 15% (*P* = 0.006) and 21% (*P* < 0.001) per 10% increment of BAL fluid neutrophil levels, respectively. The degree of BAL fluid pleocytosis was relatively low without regard to the severity of PCP. The percentage of BAL fluid neutrophils can be used as a prognostic marker in non-HIV-infected patients with PCP.

Materials and Methods

Study design, setting, and study population. This retrospective cohort study was performed at the Asan Medical Center (Seoul, Republic of Korea), a 2,700-bed tertiary care-affiliated teaching hospital with 203 intensive care unit beds and active programs for solid-organ and hematopoietic stem cell transplantation. Adult patients (aged ≥16 years) who underwent fiberoptic bronchoscopy with BAL for suspected PCP from February 2007 to April 2014 were identified by examining bronchoscopy logs and laboratory databases. Only the results from the first bronchoscopy in patients who underwent multiple bronchoscopies during a single clinical episode were included. Patients with HIV infection and those who had inadequate results of BAL cellular analysis were excluded from the analysis. The study was approved by the Asan Medical Center Institutional Review Board.

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Address correspondence to Sang-Ho Choi, sangho@amc.seoul.kr.

J.Y.L. and H.J.P. contributed equally to this article.

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Data collection and definitions. All medical records were retrospectively reviewed using standardized study protocols. Demographic characteristics, laboratory results, underlying diseases or conditions, patient management, and clinical outcomes were evaluated. On the basis of determinations of partial pressure of arterial oxygen (PaO\textsubscript{2}) measured while the subject was breathing room air or of the alveolar oxygen-arterial oxygen difference (AaDO\textsubscript{2}) prior to the first bronchoscopy, patients were grouped into those with mild (PaO\textsubscript{2} > 70 mm mercury [Hg] or AaDO\textsubscript{2} < 35 mm Hg), moderate (PaO\textsubscript{2} ≤ 70 mm Hg or AaDO\textsubscript{2} ≥ 35 mm Hg), or severe (PaO\textsubscript{2} < 60 mm Hg or AaDO\textsubscript{2} ≥ 45 mm Hg) PCP (12). Temporary use of mechanical ventilation (MV) for the purpose of bronchoscopic examination was not considered to be "MV use" to avoid overestimation of MV users. Failure of initial treatment regimen was defined as clinical deterioration during the first 5 days of treatment or lack of improvement after 7 or more days of treatment (13). Patient outcomes were in-hospital, 30-day, and 60-day all-cause mortalities, defined as deaths from all causes occurring during admission or within 30 days and 60 days, respectively, of the initial bronchoscopy that yielded a positive result for P. jirovecii.

Bronchoscopic BAL and BAL fluid processing and analysis. Fiberoptic bronchoscopy with BAL was performed according to a standardized protocol described previously (14, 15). Briefly, three consecutive aliquots (20, 30, and 30 ml) of sterile saline solution were instilled into the bronchial tree where the abnormality on the chest radiography was most apparent. In the event of bilateral diffuse infiltration, the right middle lobe or lingual segment was chosen. The fluid retrieved after first instillation was discarded, and the fluid that was subsequently retrieved was collected for analysis. A hemocytometer was used to determine total cell count. The Department of Laboratory Medicine of our institution has maintained continuous accreditation by the Accreditation Committee of the College of American Pathologists (CAP) since 1999. According to CAP Laboratory Accreditation Program hematology checklist question HEM.35340, quality control of the hemocytometer is performed after every 8 hours of patient testing using a previously assayed patient sample. The amount of BAL fluid corresponding to 10\textsuperscript{4} cells was centrifuged at 300 rpm for 5 minutes at room temperature and prepared on a microscope slide using a Thermo Shandon Cytospin instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). The slide was immediately air-dried and stained with Wright-Giemsa stain. Differential cell counting was used to determine percentages of neutrophils, lymphocytes, and alveolar macrophages.

Microbiological evaluation. From February 2007 to February 2012, direct immunofluorescence assays were performed to detect P. jirovecii in accordance with the manufacturer’s instructions, using a commercially available murine monoclonal antibody labeled with fluorescein isothiocyanate, which reacts with human and rodent Pneumocystis cysts and trophozoites (Light Diagnostics Pneumocystis carinii direct fluorescent antibody kit; Millipore, Billerica, MA, USA). From March 2012 to April 2014, we used a commercially available immunocytochemistry assay and mouse monoclonal antibody clone 3F6 (Dako Corporation, Carpinteria, CA, USA), which reacts with an antigenic epitope highly specific for P. jirovecii.

Additional microbiological evaluations were conducted according to standard procedures as previously described (14). Plate assays of BAL fluid specimens were used to quantify conventional bacteria, and for cytomegalovirus (CMV), samples were inoculated into shell vial cultures of human fetal lung fibroblasts (Diagnostic Hybrids, Inc., Athens, OH, USA), and growth was examined on days 1 and 7 by direct immunofluorescence staining.

Statistical analysis. Categorical variables were analyzed using the chi-square or Fisher’s exact test. Normally and non-normally distributed continuous variables were analyzed using Student’s t test and the Mann-Whitney U test, respectively. To evaluate the association between the neutrophil increment in BAL fluid and mortality rate, we used the linear-by-linear association test for trend determinations. Risk factors associated with mortality were assessed using univariate and multivariate logistical regression analysis. Variables with P values of less than 0.1 in the univariate analysis were included in the multivariate analysis. The final model was constructed using the stepwise selection procedure. A P value of <0.05 was considered statistically significant. The data were analyzed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographics, clinical characteristics, treatments, and outcomes. During the study period, a total of 209 patients with PCP were identified. Of these, 29 HIV-infected patients and 14 patients who had inadequate results for the BAL fluid cellular analysis were excluded, leaving a total of 166 patients with PCP in the final analysis.

Characteristics of the 166 non-HIV-infected patients with PCP are shown in Table 1. The median age was 55 years (interquartile range [IQR], 42 to 64), and 103 patients (62.0%) were men. Hematologic malignancy was the most common underlying disease (34.9%), followed by solid-organ transplantation (30.7%), solid tumor (16.3%), connective-tissue disease (9.6%), and interstitial lung disease (ILD) (9.0%). CMV coinfection was identified in 43 patients (25.9%).

PCP was classified as mild (n = 39, 23.5%), moderate (n = 7, 4.2%), and severe (n = 120, 72.3%). A comparison of patients with mild-to-moderate PCP to patients with severe PCP is presented in Table 1. Patients with severe PCP were older. There was little difference in terms of underlying disease and immunosuppressive-agent use between the two groups. In addition, the proportions of patients with CMV coinfection were not different in the two groups (23.9% versus 26.7%, P = 0.72). However, laboratory findings, such as median C-reactive protein (CRP), lactate dehydrogenase (LDH), and AaDO\textsubscript{2} levels, were significantly higher in patients with severe PCP. A higher acute physiology and chronic health evaluation (APACHE) II score was associated with severe PCP (18 versus 13, P < 0.001). At initial presentation, patients with severe PCP were more likely to receive mechanical ventilation (27.3% versus 2.2%, P < 0.001). The majority (99.4%) of patients received trimethoprim-sulfamethoxazole as an initial treatment regimen. Severe PCP was associated with higher 30-day (28.3% versus 4.3%, P = 0.001) and 60-day (43.3% versus 10.9%, P < 0.001) all-cause mortality than mild-to-moderate PCP.

BAL fluid cellular analysis. The results of BAL fluid cellular analysis are shown in Table 2. The median total white blood cell (WBC) count was 180/µl (IQR, 80 to 330) and was not associated with severity of PCP. The median percentage of neutrophils was higher in patients with severe PCP (20.4% versus 6.0%, P < 0.001), as was the absolute neutrophil count (24/µl versus 13/µl, P = 0.001). Neutrophil dominance (defined as neutrophils being the most common cell type in BAL fluid WBCs) was also significantly associated with severe PCP (29.2% versus 6.5%, P = 0.002). In contrast, the absolute alveolar macrophage count was lower in patients with severe PCP (50/µl versus 76/µl, P = 0.03). However, the median percentages of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and the CD4\textsuperscript{+}/ CD8\textsuperscript{+} T cell ratios were similar in the two groups.

Figure 1 shows the 30-day and 60-day overall mortality rates determined according to the levels of BAL fluid neutrophilia at different cutoff points (Fig. 1A) and over different ranges (Fig. 1B). In Fig. 1A, the total numbers of patients and 30-day and 60-day overall mortality rates, respectively, were as follows: for ≥10% neutrophilia (n = 94), 26% and 34%; for ≥20% neutrophilia (n = 68), 29% and 38%; for ≥30% neutrophilia (n = 46), 30% and 44%; for ≥40% neutrophilia (n = 37), 38% and 51%;
and for ≥50% neutrophilia (n = 26), 42% and 62%. Levels of 30-day and 60-day overall mortality increased at rates of 15% ($P = 0.006$) and 21% ($P < 0.001$), respectively, per 10% incremental increase of BAL fluid neutrophil levels. As shown in Fig. 1B, the total numbers of patients and the 30-day and 60-day overall mortality rates, respectively, were as follows: for <10% neutrophilia (n = 72), 17% and 24%; for 10% to 20% neutrophilia (n = 26), 15% and 23%; for 20% to 30% neutrophilia (n = 22), 27% and 27%; for 30% to 40% neutrophilia (n = 9), 0% and 11%; for 40% to 50% neutrophilia (n = 11), 27% and 27%; and for ≥50% neutrophilia (n = 16), 42% and 62%. The levels of 30-day and 60-day overall mortality increased at rates of 29% ($P < 0.001$) and 32% ($P < 0.001$), respectively, per 10% incremental increase of BAL fluid neutrophil levels.

**Risk factors associated with 30-day and 60-day mortalities.**

In the univariate analysis, age, male gender, hematologic malignancy, solid-organ transplantation, CRP and LDH levels, APACHE II score, mechanical ventilation at initial presentation, and percentage of BAL fluid neutrophils were identified as significant variables associated with mortality and were included in the final model. The results of multiple-logistic-regression analysis of predictors for 30-day and 60-day mortalities are summarized in Table 3. Male gender (adjusted OR [aOR], 2.52; 95% confidence interval [CI], 1.10 to 5.75; $P = 0.03$), solid-organ transplantation
TABLE 2 Cellular profiles of bronchoalveolar lavage fluid in 166 non-HIV-infected patients with Pneumocystis jirovecii pneumonia\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All (n = 166)</th>
<th>Mild to moderate (n = 46)</th>
<th>Severe (n = 120)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC count (cell/(\mu l))</td>
<td>180 (80–330)</td>
<td>230 (130–355)</td>
<td>180 (80–300)</td>
<td>0.32</td>
</tr>
<tr>
<td>Neutrophil %, median (IQR)</td>
<td>13.1 (5.3–36.2)</td>
<td>6.0 (3.4–12.9)</td>
<td>20.4 (5.8–40.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lymphocyte %, median (IQR)</td>
<td>31.7 (11.8–55.4)</td>
<td>45.3 (19.9–59.8)</td>
<td>32.1 (13.0–54.6)</td>
<td>0.12</td>
</tr>
<tr>
<td>Alveolar macrophage %, median (IQR)</td>
<td>30.2 (18.2–54.2)</td>
<td>40.7 (22.0–57.1)</td>
<td>28.3 (15.4–39.8)</td>
<td>0.01</td>
</tr>
<tr>
<td>Neutrophil count (cell/(\mu l), median (IQR))</td>
<td>22 (6–59)</td>
<td>13 (4–30)</td>
<td>24 (7–73)</td>
<td>0.001</td>
</tr>
<tr>
<td>Lymphocyte count (cell/(\mu l), median (IQR))</td>
<td>44 (12–143)</td>
<td>68 (29–210)</td>
<td>49 (8–144)</td>
<td>0.17</td>
</tr>
<tr>
<td>Alveolar macrophage count (cell/(\mu l), median (IQR))</td>
<td>51 (19–112)</td>
<td>76 (36–125)</td>
<td>50 (13–96)</td>
<td>0.03</td>
</tr>
<tr>
<td>Neutrophils dominant(^b)</td>
<td>38 (22.9)</td>
<td>3 (6.5)</td>
<td>35 (29.2)</td>
<td>0.002</td>
</tr>
<tr>
<td>Lymphocytes dominant(^b)</td>
<td>76 (45.8)</td>
<td>24 (52.2)</td>
<td>52 (43.3)</td>
<td>0.31</td>
</tr>
<tr>
<td>Alveolar macrophages dominant(^b)</td>
<td>52 (31.3)</td>
<td>19 (41.3)</td>
<td>33 (27.5)</td>
<td>0.09</td>
</tr>
<tr>
<td>CD4(^+) T cell %, median (IQR)</td>
<td>33.4 (23.4–41.3)</td>
<td>34.4 (23.5–50.8)</td>
<td>33.0 (23.3–39.8)</td>
<td>0.21</td>
</tr>
<tr>
<td>CD8(^+) T cell %, median (IQR)</td>
<td>49.6 (32.1–59.8)</td>
<td>50.3 (29.4–59.6)</td>
<td>49.0 (32.7–60.4)</td>
<td>0.74</td>
</tr>
<tr>
<td>CD4(^+)/CD8(^+) T cell ratio, median (IQR)</td>
<td>0.7 (0.4–1.1)</td>
<td>0.7 (0.4–1.5)</td>
<td>0.7 (0.4–1.0)</td>
<td>0.74</td>
</tr>
</tbody>
</table>

\(^a\) Data are no. (%) of patients unless indicated otherwise. WBC, white blood cell; IQR, interquartile range.
\(^b\) The most common cell type among bronchoalveolar lavage fluid WBCs.

The results of this study showed that BAL fluid neutrophilia in non-HIV-infected patients with PCP was associated with severe disease and higher mortality. It is thought that severe pneumocystis pneumonia is characterized by neutrophilic lung inflammation that may result in diffuse alveolar damage, impaired gas exchange, and respiratory failure (1). Limper et al. suggested that poor clinical outcomes were more closely related to the degree of lung inflammation than to the organism burden in PCP (22). This implies that measuring the degree of neutrophilia in BAL fluid may play a role in predicting clinical outcome. A few investigators have suggested that BAL fluid neutrophilia (>5% to 10%) can be a useful marker for predicting poor clinical course and outcome, such as the use of mechanical ventilation and death, in HIV-infected patients with PCP (6–9). Very limited information is available on the prognostic utility of BAL fluid cellular analysis in non-HIV-infected patients with PCP. A French group and a Japanese group, studying 39 and 29 patients, respectively, found that BAL fluid neutrophilia (>15% to 30%) might be a significant prognostic factor associated with fatal outcome (10, 11). In the current...
study, we included a relatively large number of cases with enhanced statistical power compared to that of previous studies and provided detailed data on the association between neutrophil incremental increases in BAL fluid and mortality rates according to different cutoff points.

Although neutrophilia appears to be a useful marker of lung damage during pneumocystis infection, its role in the pathogenesis of PCP remains unclear. It has been proposed that severe respiratory failure in PCP may be related to neutrophil-mediated inflammatory processes resulting in early-stage acute respiratory distress syndrome (8, 23). However, in a recent animal study, there were no significant differences in terms of pulmonary damage between knockout mice with neutrophil dysfunction and comparable wild-type mice, showing that neutrophils might not

TABLE 3 Multiple logistic-regression analysis of predictors for 30-day and 60-day mortalities in non-HIV-infected patients with Pneumocystis jirovecii pneumonia

<table>
<thead>
<tr>
<th>Predictor</th>
<th>30-day mortality</th>
<th>60-day mortality</th>
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<tbody>
<tr>
<td></td>
<td>aOR (95% CI)</td>
<td>aOR (95% CI)</td>
</tr>
<tr>
<td>Male gender</td>
<td>2.52 (1.10–5.75)</td>
<td>0.28 (0.09–0.87)</td>
</tr>
<tr>
<td>Solid-organ transplantation</td>
<td>1.08 (1.01–1.16)</td>
<td>1.02 (1.01–1.03)</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>1.09 (1.03–1.17)</td>
<td>1.02 (1.01–1.04)</td>
</tr>
<tr>
<td>BAL fluid neutrophil %</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

aOR, adjusted odds ratio; CI, confidence interval; APACHE, acute physiology and chronic health evaluation; BAL, bronchoalveolar lavage.
be the causative agent of tissue damage (24). Therefore, further research is warranted to determine the exact role of neutrophils in the pathogenesis of PCP.

Through multivariate analysis, we found that solid-organ transplantation was significantly associated with a better outcome. Previous reports have shown that mortality rates vary according to underlying disease or condition among non-HIV-infected patients with PCP (3–5). A Dutch group reported that the overall mortality rate was 35% among 78 HIV-negative patients, with the lowest rate (8%) among 13 kidney transplant recipients (25). In the present study, the 30-day mortality rates in transplantation were low regardless of transplant organ type (kidney, 11.8% [4/34]; liver, 12.5% [1/8]; heart, 0% [0/4]; simultaneous pancreas-kidney, 0% [0/4]; pancreas, 0% [0/1]) compared with those seen with other underlying diseases. Factors that contribute to differences in mortality rates among different disease groups in non-HIV-infected patients with PCP should be further investigated.

This study had several limitations. First, the retrospective design and single-center site were the main limitations. Second, we could not establish the organism burden in our PCP patients because the number of P. jiroveci in BAL fluid samples had not been determined. Third, concomitant bacterial or viral infection in patients with PCP might result in BAL fluid neutrophilia and act as a confounding factor in interpreting our results. However, the median percentages of neutrophils were not different in patients with and without bacterial coinfection (14.5% versus 13.0%, P = 0.42), because there were only 6 patients (3.6%) coinfected with bacteria. In addition, although we found that patients with CMV coinfection had a higher median percentage of neutrophils than patients without CMV coinfection (25.1% versus 10.9%, P = 0.02), the proportions of patients with CMV coinfection were not different in the mild-to-moderate PCP group and the severe PCP group, and the median percentage of neutrophils was higher in patients with severe PCP, regardless of the presence of CMV coinfection (see Table S1 in the supplemental material). Furthermore, CMV coinfection was not associated with increased levels of 30-day and 60-day all-cause mortalities (see Table S2). This suggests that bacterial and CMV coinfections are unlikely to have influenced our findings. Finally, while Fig. 1A shows a sustained increase in the overall mortality rate as the cutoff points increase by 10% increments, the results for some of the ranges of BAL fluid neutrophilia shown in panel B, especially for 30% to 40% neutrophilia, did not follow this trend, possibly because of the small numbers of patients falling in these ranges.

In conclusion, regardless of severity, the degree of BAL fluid pleocytosis is relatively low in PCP. The percentage of BAL fluid neutrophils may be used as a prognostic marker in non-HIV-infected patients with PCP. Our study results suggest that clinicians could make use of BAL fluid cellular profiles to identify potential patients at risk of poor outcome and to make therapeutic decisions, such as choosing the appropriate site of care or the level of patient monitoring.

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We declare that we have no potential conflicts of interest.

REFERENCES


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