Immunological Characterization of Bronchoalveolar Lavage Fluid in Patients With Acute Pulmonary Coccidioidomycosis

Lance A. Nesbit,1,3 Kenneth S. Knox,7 Chinh T. Nguyen,2,4 Justin Roesch,5 L. Joseph Wheat,6 Suzanne M. Johnson,7 Demosthenes Pappagianis,7 Suzette Chavez,1 and Neil M. Ampel2,3

1Research Services, 2Medical Services, Southern Arizona Veterans Affairs Health Care System, 3Section of Pulmonary, Allergy, Critical Care, and Sleep Medicine, 4Division of Infectious Diseases, Department of Medicine, and 5Internal Medicine Residency Program, Arizona Health Sciences Center, University of Arizona, Tucson; 6Miravista Diagnostics, Indianapolis, Indiana; and 7Department of Medical Microbiology and Immunology, University of California at Davis

Background. The specific cellular immunological characteristics of bronchoalveolar lavage (BAL) fluid in acute pulmonary coccidioidomycosis have not been defined.

Methods. BAL fluid from patients living in a coccidioidomycosis-endemic region of Arizona who were undergoing bronchoscopy because of pulmonary infiltrates was analyzed. Mononuclear cells from BAL fluid and peripheral blood mononuclear cells (PBMCs) were incubated with the coccidioidal antigen T27K in vitro, and cellular immunological assays were performed.

Results. Forty-six patients were studied. Twelve received a diagnosis of acute pulmonary coccidioidomycosis, 17 received other diagnoses, and 17 had no diagnosis established. There was an increased proportion of polyfunctional CD8+ T cells after antigen stimulation from subjects with coccidioidomycosis as compared to those with another diagnosis (P = .025). In cells collected from BAL fluid and in PBMCs, the concentrations of interferon γ, tumor necrosis factor α, and interleukin 17 (IL-17) were all significantly increased in samples from those with acute pulmonary coccidioidomycosis, compared with the other 2 groups (for all, P < .05).

Conclusions. When incubated in vitro with a coccidioidal antigen preparation, cells from both BAL fluid and peripheral blood obtained from patients with pulmonary coccidioidomycosis demonstrated specific cellular immune responses, including expression of IL-17.

Keywords. coccidioidomycosis; human; cellular immunity; bronchoalveolar lavage.

It is estimated that there are 150,000 cases of coccidioidomycosis in the United States annually [1], with the vast majority of these acquired by inhalation. Among these infections, 40% result in a symptomatic pulmonary syndrome, often mimicking bacterial community-acquired pneumonia [2, 3].

Bronchoalveolar lavage (BAL), a technique for obtaining fluid from the pulmonary bronchoalveolar lining, has been developed over the past several decades both for clinical and research purposes [4, 5]. BAL fluid is analyzed to ascertain the local immunological response to infection. In pulmonary tuberculosis, multiple reports have demonstrated an increased cellular immunological response in BAL fluid [6–8]. There have been several clinical studies of the role of BAL in the diagnosis of coccidioidomycosis [9–11], but all were published more than a decade ago, and none have examined the specific immunological response to coccidioidal infection.

We prospectively obtained BAL fluid from patients living in a coccidioidomycosis-endemic region of Arizona who underwent bronchoscopy because of pulmonary infiltrates. In addition to analysis of BAL fluid, we incubated mononuclear cells from both BAL fluid and peripheral blood with a coccidioidal antigen preparation to assess the specific in vitro cellular immune response
among patients with and those without a clinical diagnosis of coccidioidomycosis. In this article, we describe the clinical and immunological attributes of BAL fluid from patients with a definite diagnosis of acute pulmonary coccidioidomycosis and compare these results to those for subjects with pulmonary infiltrates due to other etiologies and to those for subjects for whom no diagnosis was determined.

**METHODS**

**Human Subjects**

Subjects at the University of Arizona Medical Center or the Southern Arizona Veterans Affairs Health Care System (SAVAHCS) who were older than 18 years were referred for BAL because of an acute pulmonary lesion. Coccidioidal serologic analysis was performed in the clinical laboratory by use of an immunodiffusion tube precipitin (IDTP) and immunodiffusion complement fixation (IDCF) test or a commercial enzyme immunoassay (ELIA). BAL fluid and peripheral blood specimens were collected after patients provide informed consent, and all experiments were performed according to specifications of and with approval from the Human Subjects Protection Program of the University of Arizona.

**Antigen Preparation and Peripheral Blood Mononuclear Cell (PBMC) Isolation**

T27 K was prepared as previously described [12]. Briefly, a continuous culture of Silveira strain Coccidioides posadasii spherules were mechanically disrupted and centrifuged at 27 000 × g. The soluble portion was collected, preserved with thimerosal, and lyophilized. After the lyophilate was weighed, it was diluted in phosphate-buffered saline (PBS; Invitrogen Life Sciences, Carlsbad, CA) and sterilized by passage through a 0.2-µm filter (Millipore, Billerica, MA). T27 K was used at a concentration of 20 µg/mL in all experiments. Blood samples were obtained by venipuncture, and PBMCs were isolated as previously described [13].

**Performance of BAL and Analysis of BAL Fluid**

BALs were performed as previously described [14]. Because the procedures were done for clinical purposes, the exact method was dictated by the clinical scenario and physician preference. In all cases, the upper airway was anesthetized with 2% topical lidocaine, and a fiberoptic bronchoscope was passed into the subsegmental bronchi of the most affected lung region. Either 4–6 25-mL aliquots or 2–3 50-mL aliquots of normal saline stored at room temperature were instilled into lung subsegments. Gentle continuous suction was applied after each aliquot was instilled, to recover the fluid. Once the fluid specimen was obtained, it was transferred from the collection vial to a 50-mL conical tube (Becton Dickinson; Franklin Lakes, NJ). Fluid recovered from the lung was kept on ice until processed. The samples were centrifuged for 5 minutes at 500 × g, and the supernatant was stored frozen until antigen testing. Cells obtained from the procedure were used immediately for stimulation assays. Approximately 5–30 × 10⁶ cells were obtained from each bronchoscopy. >95% of which were mononuclear cells. A total of 4×10⁴ cells were centrifuged onto Shandon single-well cytospin slides, using a Cytospin 4 centrifuge (Thermo Scientific). The slides were air dried and stained with Wright-Giemsa stain. Cell differentials of lymphocytes and eosinophils were counted on the basis of morphological criteria.

**BAL Cells and PBMC Incubation**

Bronchoalveolar cells at a concentration of 1.5 × 10⁶ viable cells and PBMCs at a concentration of 2 × 10⁶ cells were resuspended in 2.0 mL of AIM-V serum-free medium (Invitrogen, Carlsbad, CA) and incubated in a 5% CO₂ humidified atmosphere alone or with 10 µg/mL T27 K. In addition, samples were also incubated with 1 µg/mL of the superantigen staphylococcal enterotoxin B (Sigma Chemical, St. Louis, MO) or 5 µg/mL of phytohemagglutinin (Sigma) as a positive control. During the final 12 hours of incubation, 10 µg/mL brefeldin A (eBioscience) was added to the culture. After incubation, the cells were centrifuged at 500 × g. The cell supernatant was retained for cytokine analysis by enzyme-linked immunosorbent assay (ELISA), whereas the pellet was resuspended in Dulbecco’s modified phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) and analyzed by flow cytometry.

**Intracellular Cytokine Analysis**

After incubation, nonadherent cells from BAL fluid and PBMCs were stained with fluorochrome-conjugated antibodies specific for CD3, CD4 or CD8, CD69, interleukin 2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α; BD Biosciences, San Jose, CA). Cells were stained in PBS for 30 minutes at 4°C, using surface-expressed antibodies. Afterward, the cells were fixed and permeabilized using the Fix and Perm Kit according to the manufacturer’s instructions (Caltag, Burlingame, CA). Finally, cells were stained for 30 minutes at 4°C for detection of intracellular cytokines. Cells were analyzed using a FACSCanto II (Becton Dickinson, San Jose, CA).

For analysis of cytokine frequencies, nonadherent cells were analyzed by flow cytometry, using a Boolean gating strategy. Analysis was performed using FlowJo software (Tree Star, Ashland, OR), with functionality determined through cytokine-expressing cells of CD3⁺ T lymphocytes. Subsequent analysis was performed using Simplified Presentation of Incredibly Complex Evaluations (SPICE), version 5.22 (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health). All values for comparison of polyfunctional cytokine expression were subtracted from the values for cells not incubated with T27 K antigen preparation.
Supernatant Cytokine Analysis
Cytokine supernatant concentrations were determined by ELISA. For IL-2, IFN-γ, and TNF-α, the human OptEIA kits from BD Biosciences (San Jose, CA) were used. For determination of interleukin 17 (IL-17) supernatant concentrations, the human IL-17 kit from R&D Systems (San Diego, CA) was used. All assays were run according to the manufacturer’s instructions. Sample results were compared to a standard curve generated from a recombinant cytokine protein provided with each kit.

Statistical Analysis
Results are expressed as medians with minimum and maximum values for the subject group. Because data were not normally distributed, statistical analyses for continuous data were performed using the Kruskal-Wallis and Mann-Whitney U tests. For categorical data, the Pearson χ² test was used.

RESULTS

Subject Description and Characterization
A total of 46 subjects were entered into the study and divided into 3 clinical groups on the basis of their eventual pulmonary diagnosis. Twelve subjects received a diagnosis of acute primary pulmonary coccidioidomycosis. This was based either on growth of Coccidioides on culture of a respiratory sample or by a positive coccidioidal serologic test in the context of a compatible clinical syndrome. The median age of this group was 55.5 years. Seven were males, and 5 were females. Ten were white and non-Hispanic, and 2 were white and Hispanic. Five subjects had underlying immunosuppression, including 1 with psoriatic arthritis, who was receiving adalimumab; 1 with breast cancer, who was receiving chemotherapy; 1 who received a cardiac transplant; 1 with chronic liver disease due to hepatitis C virus; and 1 with chronic lymphocytic leukemia; 1 with bladder cancer, who was receiving gemcitabine; and 1 with metastatic adenocarcinoma.

Ten of the 12 subjects had a positive result of coccidioidal serologic analysis at the time of BAL. Of these 12 subjects, IDTP antibody was found in 5 of 6. No sera were concentrated prior to testing. In addition, EIA was positive for immunoglobulin M (IgM) for 3 of 6 subjects, and immunoglobulin G (IgG) was detected in 3 of 6. EIA findings for IgM and IgG were discordant in 4 instances. In the 8 patients for whom IDCF was titered, the ranged from <1:2 to 1:4.

Seventeen subjects had a diagnosis other than coccidioidomycosis. These included 1 each with tuberculosis, metapneumovirus pneumonia, respiratory syncytial virus pneumonia, and bronchopulmonary aspergillosis. Four patients received a diagnosis of sarcoidosis, and 9 had malignancy. The median age was 64 years and included 13 men and 3 women. All subjects were white and non-Hispanic. Five subjects had underlying immunosuppression, including 1 who received a renal transplant; 1 with sarcoidosis, who was receiving prednisone; 1 with chronic lymphocytic leukemia; 1 with bladder cancer, who was receiving gemcitabine; and 1 with metastatic adenocarcinoma. None of the 8 subjects tested had a positive result of a coccidioidal serologic test.

There was another 17 subjects without a diagnosis established by BAL, including 3 with previously diagnosed coccidioidomycosis. One subject in this group had a later diagnosis of usual interstitial pneumonitis established by video-assisted thorascopic biopsy. The median age in this group was 64 years, and all were men. Thirteen were white and non-Hispanic, 3 were white and Hispanic, and 1 was Native American. Four subjects had underlying immunosuppression, including 1 with Waldenstrom disease; 1 with asthma, who was receiving prednisone; 1 who received a renal transplant; and 1 with rheumatoid arthritis, who was receiving azathioprine and infliximab. None of the 13 subjects who were tested had a positive result of a coccidioidal serologic test.

The clinical characteristics for the 3 diagnostic groups are displayed in Table 1. There was a significant difference only in the male-to-female ratio between the groups, with significantly more females in the group who received a diagnosis of acute pulmonary coccidioidomycosis (P = .018). The median follow-up duration for the 39 subjects for whom this information was available was 311 days (range, 7–1151 days). Among those with

Table 1. Clinical Characteristics of 46 Subjects Who Underwent Bronchoalveolar Lavage for an Undiagnosed Pulmonary Infiltrate

<table>
<thead>
<tr>
<th>Variable</th>
<th>Acute Pulmonary Coccidioidomycosis (n = 12)</th>
<th>Other Diagnoses (n = 17)</th>
<th>No Diagnosis (n = 17)</th>
<th>P²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, median (range)</td>
<td>55.5 (26–79)</td>
<td>66 (37–84)</td>
<td>64 (23–84)</td>
<td>.165²</td>
</tr>
<tr>
<td>Male: Female</td>
<td>7:5</td>
<td>13:4</td>
<td>17:0</td>
<td>.018</td>
</tr>
<tr>
<td>Race, ethnicity</td>
<td></td>
<td></td>
<td></td>
<td>.268</td>
</tr>
<tr>
<td>White, non-Hispanic</td>
<td>10</td>
<td>17</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>White, Hispanic</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Immuno compromised</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>.575</td>
</tr>
</tbody>
</table>

² Based on the Pearson χ² test, unless otherwise indicated.
² Based on the Kruskal-Wallis test.
coccidioidomycosis, 8 of 10 were alive. One died rapidly due to overwhelming pulmonary coccidioidomycosis 1 week after BAL. Coccidioidal infection in the other 9 subjects resolved, but 1 died of a metastatic malignancy 110 days after BAL. Of the 29 other subjects without diagnosed coccidioidomycosis during follow-up, 4 were later found to have died.

Clinical Characterization of BAL Fluid
Table 2 depicts the clinical characterization of BAL fluid on the basis of subject group, including findings of cytology, fungal culture, and tests for detection of antigen. There was no significant difference in either the median percentage of lymphocytes (P = .622) or the median percentage of eosinophils in BAL fluid (P = .354) between subjects with coccidioidomycosis and those with other diagnoses or no diagnosis.

A fungal culture of BAL fluid from 5 of the 12 patients with a diagnosis of acute primary pulmonary coccidioidomycosis grew *Coccidioides*. In 1 of the 7 with a negative result of BAL fluid culture, sputum culture was positive for *Coccidioides* of the 32 subjects without a diagnosis of coccidioidomycosis for whom fungal culture of BAL fluid was performed, none tested positive for *Coccidioides* on culture. Two subjects did not have a culture performed. In no instance was *Coccidioides* seen as a spherule on histological examination, either by potassium hydroxide or Papanicolaou staining.

BAL fluid from 32 subjects was tested for coccidioidal galactomannan antigen after treatment with ethylenediaminetetraacetic acid [15]. Among the 12 subjects with confirmed coccidioidomycosis, results of the galactomannan assay were positive for 3, with concentrations of 0.08, 0.20, and 0.31 ng/mL. These positive assays occurred for 2 subjects for whom culture of BAL fluid was positive for *Coccidioides* and in 1 additional subject for whom culture of BAL fluid was negative for *Coccidioides* but a contemporaneous sputum culture grew *Coccidioides*. In all other cases, there was no detectable galactomannan antigen.

Immunological Comparison of Samples From Subjects With and Subjects Without a Diagnosis of Acute Pulmonary Coccidioidomycosis
We next examined the differences in the specific coccidioidal immunological response between subjects with and subjects without a diagnosis of acute pulmonary coccidioidomycosis. First, we examined the simultaneous intracellular expression of IL-2, IFN-γ, and TNF-α by CD4+ and CD8+ T lymphocytes after in vitro incubation with the coccidioidal antigen preparation T27 K among mononuclear cells isolated from the BAL fluid.
fluid and PBMCs. The median percentage increase in polyfunctional CD4+ T lymphocytes produced in response to T27 K stimulation, relative to production in unstimulated controls in the BAL fluid compartment, was 0.49% for subjects with a diagnosis of acute pulmonary coccidioidomycosis, compared with 0.06% for those with either no diagnosis or another diagnosis \((P = .289)\); for CD8+ T cells, the increase was 1.13%, compared with 0.08% \((P = .006)\). For the peripheral blood compartment, the median percentage increase in polyfunctional CD4+ T lymphocytes among subjects with coccidioidomycosis was 0.04%, compared with 0.01% among those with another or no diagnosis \((P = .045)\), whereas the percentage increases for CD8+ T lymphocytes were 0.04% and 0.02%, respectively \((P = .240)\).

SPICE analysis depicting the proportion of CD4+ and CD8+ T lymphocytes producing 1, 2, or 3 intracellular cytokines in response to T27 K antigen, compared with unstimulated cells, in the BAL fluid and peripheral blood compartments is shown in Figure 1. The proportion of polyfunctional BAL CD8+ T cells from subjects with acute pulmonary coccidioidomycosis was significantly increased, compared to those with no diagnosis \((P = .025)\), but there were significantly more T27K-responsive polyfunctional PBMC CD4+ T cells from donors with no diagnosis, compared with those with acute pulmonary coccidioidomycosis \((P = .005)\).

We also examined the release of cytokines into the supernatant fluid of cultured mononuclear cells obtained from BAL fluid after in vitro incubation with T27 K (Figure 2). Concentrations of IL-2, IFN-γ, TNF-α, and IL-17 were all significantly increased in cultures containing mononuclear cells from BAL fluid from subjects with acute pulmonary coccidioidomycosis, compared with subjects with other diagnoses \((P < .05 \text{ for all 4 cytokine comparisons})\), and IL-2, TNF-α, and IL-17 levels were all significantly increased in samples from subjects with acute pulmonary coccidioidomycosis, compared with subjects with no diagnosis \((P < .05 \text{ for all 3 cytokine comparisons})\).

As shown in Figure 3, concentrations of IFN-γ, TNF-α, and IL-17 were all significantly increased in PBMC samples from subjects with acute pulmonary coccidioidomycosis, compared with those with another diagnosis \((P < .05 \text{ for all 3 cytokine comparisons})\), whereas IL-2, IFN-γ, TNF-α, and IL-17 levels were all significantly increased in donors with acute pulmonary coccidioidomycosis, compared with those with no diagnosis \((P < .05 \text{ for all 4 cytokine comparisons})\). Release of IFN-γ and IL-17 appeared to be linked. In 3 of 8 subjects with acute pulmonary coccidioidomycosis, levels of IFN-γ and IL-17 in the BAL compartment were <50 and <10 ng/mL, respectively. In the other 5 cases, both IFN-γ and IL-17...
levels were greater than these values ($P = .005$). Similar results were observed in the peripheral blood (data not shown).

**DISCUSSION**

This is the first study to compare the specific cellular immune response in the BAL fluid and peripheral blood compartments among patients with acute pulmonary coccidioidomycosis and is the first report of a case series involving BAL and patients with pulmonary coccidioidomycosis in over a decade [9, 10, 16]. We were able to compare the results for subjects with acute pulmonary coccidioidomycosis to those without this diagnosis. We divided the latter subjects into 2 groups, those with another diagnosis and those without any diagnosis, since the latter made up a large proportion of the total number of cases and may have differed from those with an established diagnosis.

Using in vitro incubation of mononuclear cells from both BAL fluid and peripheral blood with the coccidioidal antigen preparation T27 K, we found that both compartments demonstrated specific cellular immune responses among subjects with a diagnosis of coccidioidal pneumonia but not in individuals without that diagnosis. We previously demonstrated that T27 K acts to specifically elicit a T-helper cell type 1 (Th1) cellular immune response in PBMCs from donors with resolved coccidioidomycosis, including inducing polyfunctional CD4$^+$ T lymphocytes [13]. In the present study, we found significantly increased numbers of polyfunctional CD8$^+$ T lymphocytes in BAL fluid from subjects with acute pulmonary coccidioidomycosis, compared with those in the other groups. SPICE analysis also demonstrated an increased proportion of polyfunctional CD8$^+$ T cells in subjects with acute pulmonary coccidioidomycosis. There was an increased number and proportion of CD4$^+$ T lymphocytes in the peripheral blood after antigen stimulation among donors with no diagnosis, compared with subjects with acute pulmonary coccidioidomycosis. These results could suggest that these donors without a diagnosis of coccidioidomycosis may have been previously infected by *Coccidioides*. The limited number of polyfunctional T cells detected in the peripheral blood of subjects with pulmonary coccidioidomycosis may be the result of the acuteness of the infection in these patients. This is supported by our previous data demonstrating that antigen-responsive polyfunctional CD4$^+$ T cells reside in the peripheral blood of individuals with clinically resolved coccidioidomycosis [13].

In both the BAL fluid and peripheral blood compartment, we found that antigen-induced release of IL-2, IFN-γ, TNF-α,
and IL-17 was significantly increased among subjects with acute pulmonary coccidioidomycosis, compared with the other groups. In murine studies of coccidioidomycosis, IL-17-producing cells in the lung have been associated with vaccine-induced protection [17, 18]. We have not observed the release of IL-17 after antigen incubation of PBMCs obtained from donors with resolved coccidioidomycosis (unpublished observations), suggesting that the release of IL-17 by cells in the BAL fluid and blood compartment after antigen stimulation could represent an early immunologic response that might serve as a clinical marker for acute pulmonary coccidioidomycosis.

This study had several limitations. First, it surveyed a relatively small population. Moreover, the decision to perform BAL was made clinically, which may have led to unrealized biases. In addition, analysis of BAL fluid is confounded because of the variable yield and number of cells obtained. BAL fluid represents only about 3% of the lung compartment and may not be reflective of the global pulmonary immunological reaction. Despite this, we found that specific immunological characteristics, particularly antigen-induced IL-17 release, was associated with acute coccidioidal pneumonia. While our results clearly demonstrate a difference in the coccidioidal cellular immune response in the BAL compartment among subjects with acute pulmonary coccidioidomycosis as compared to other groups, these data are not robust enough to establish this as a diagnostic tool.

Notes

Acknowledgments. We thank Jason Brenchley for his assistance with SPICE analysis.

Financial support. This work was supported by the Department of Medicine, University of Arizona.

Potential conflicts of interest All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References


