Bronchoalveolar Lavage Findings in Patients Seropositive for the Human Immunodeficiency Virus (HIV)*

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To evaluate bronchoalveolar lavage (BAL) findings in patients infected with human immunodeficiency virus (HIV), 39 patients seropositive for the virus but with no history of opportunistic infection were studied. Opportunistic organisms such as *Pneumocystis carinii* were not found in any of the 35 BAL fluids sent for special stains and cultures. Three of 16 (18 percent) BAL fluids sent for HIV culture were positive compared with a 60.9 percent blood HIV culture positivity in the same group. To evaluate cellular recovery, the patients were divided into Walter Reed (WR) groups 1 and 2 (blood CD4 \(\geq 400\) cu mm) and WR3 to WR5 (blood CD4 <400/cu mm). Compared with ten nonsmoking healthy controls, the WR1 and WR2 group had a greater overall cellular recovery but this was not statistically significant when the smokers were excluded. There was no difference in macrophage or lymphocyte percentages in either patient group compared with controls. T-cell subset analysis of a small group of WR1 to WR5 patient BAL fluids revealed no difference in CD4 numbers or the CD4/CD8 ratio between WR1 and WR2 and WR3 to WR5 patients. We conclude that opportunistic pulmonary infection is unlikely in HIV-seropositive patients with normal chest roentgenograms despite symptoms of dyspnea on exertion. Also, HIV can be isolated from BAL fluid from these patients although not as often as from blood. Finally, there appears to be no distinct progression in BAL cellular findings before the onset of acquired immunodeficiency syndrome.

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**WR** = Walter Reed; **GMS** = Gomori-methenamine-silver; **PBS** = phosphate-buffered saline solution; **PBL** = peripheral blood lymphocyte; **PHA** = phytohemagglutinin; **ELISA** = enzyme-linked immunoabsorbent assay; **CMV** = cytomegalovirus

The human immunodeficiency virus (HIV) currently has infected an estimated 1 to 2 million Americans. Most of the natural history of the disease occurs before the onset of opportunistic infection or malignant neoplasm—a period of up to eight or more years. During this period, the patient may be asymptomatic or seek medical attention for such problems as adenopathy or oral candidiasis. Within the US military forces, mandatory serologic screening for HIV antibody has identified a population at various stages of HIV infection. At our own institution, we have seen a number of these patients who have complained of a decline in their ability to perform physical training exercise at a time when history, physical examination, and chest roentgenogram revealed no evidence for overt opportunistic infection. Because of these patients, we undertook a study to examine them for the cause of their symptoms. One possible cause that we considered for the dyspnea was early infection with *Pneumocystis carinii*. Because patients with acquired immunodeficiency syndrome (AIDS) treated for *P. carinii* pneumonia may continue to harbor the organism after resolution of roentgenographic and physical signs, we reasoned that our own patients might in fact harbor the organism or other pathogens before the onset of such signs. To evaluate for opportunistic infection, bronchoalveolar lavage (BAL) was selected because of its high sensitivity in the AIDS population and because of its low morbidity.

We saw this as an opportunity to simultaneously study BAL fluid immunology in various stages of HIV infection as well as to determine the BAL HIV culture positivity rate. Much of the previous work in this area has been done in patients with AIDS. These patients allowed us to study these factors without the potentially confounding variable of overt pulmonary opportunistic infection. Many of these patients underwent exercise testing and gallium scanning. These results are reported elsewhere.
Materials and Methods

Study Population

Patients referred for either initial or follow-up evaluation of HIV seropositivity between July 1987 and November 1988 were interviewed, and consenting individuals not meeting exclusion criteria were enrolled. A particular effort was made to enroll patients with any complaints of dyspnea on exertion or difficulty with exercise. Patients were excluded for the following reasons: (1) preliminary evaluation revealing obvious pulmonary infection; (2) a history of or symptoms suggestive of atherosclerotic heart disease or other significant cardiac disease; (3) a history of asthma requiring medication; (4) other preexisting pulmonary disease; and (5) other significant complicating medical disorders.

The study was reviewed and approved by the institutional review board. Informed consent was obtained from all subjects.

All patients completed a questionnaire that included dyspnea questions from the American Thoracic Society Epidemiology Standardization Project, and a modified Borg scale was used to quantify the level of dyspnea with moderate exertion at the time of the study compared with similar exertion in previous years. Each patient had blood drawn for a complete blood cell count, lymphocyte subset analysis, and in some cases HIV culture. An anergy panel was applied and based on the results and physical examination, the patient's Walter Reed (WR) stage was defined. The BAL findings of these patients were compared with those of ten healthy HIV seronegative nonsmoking controls and nine patients with AIDS (WR6) undergoing BAL during the study period. Briefly, the WR classification is as follows: WR1—HIV seropositive, otherwise normal; WR2—HIV seropositive with chronic lymphadenopathy, CD4 ≥400/μL; WR3—CD4 <400/μL but normal delayed hypersensitivity; WR4—CD4 <400/μL with partial anergy; WR5—CD4 <400/μL with either complete anergy or oral thrush; and WR6—opportunistic infection present. The anergy panel included intradermal injection of antigens of mumps, Trichophyton, tetanus, and Candida as well as a Mantoux skin test.

BAL Fluid

Fiberoptic bronchoscopy was performed in the sitting position by either the transnasal or transoral route after topical anesthesia with 2% lidocaine. The tip of the instrument was then advanced and wedged into a segmental or subsegmental bronchus in the right middle lobe. Sterile 0.9% saline solution was infused in 50-μL aliquots for a total of 200 to 300 μL; the fluid was recovered by gentle aspiration after each aliquot.

The lavage fluid was divided for subsequent analysis. Three milliliters were sent for cytologic analysis. The specimen was centrifuged and the pellet was fixed to slides. Comometha-mamine-silver (GMS), acid-fast bacillus (AFB), and Papanicolaou stains were read by a cytopathologist. Three milliliters were sent for routine bacterial culture (held seven days to allow for growth of Nocardia), as well as mycobacterial and fungal culture. Five milliliters were frozen at −70°C overnight for HIV cultures.

The remaining fluid were filtered through a single layer of gauze and a 1-μL aliquot was removed for total cell count via hemocytometer and a differential cell count on 200 to 500 cells. In some cases, the rest of the fluid was sent for lymphocyte subset analysis. These fluids were brought to final glucose and electrolyte concentrations of Hanks balanced salt solution by slowly pouring in a concentrated solution with the appropriate ingredients with constant swirling.

Lymphocyte Subset Analysis

Peripheral Blood: Each specimen was drawn in a tube containing edetic acid for a complete blood cell count and absolute lymphocyte count. Lyophilized monoclonal antibodies to human lymphocyte antigens labeled with fluorescein isothiocyanate (Coulter Immunology, Hialeah, Fla) were reconstituted as recommended by the manufacturer with phosphate-buffered saline solution (PBS). Two controls were also made. One control was a similar concentration of mouse IgG1 to evaluate for nonspecific antibody binding, and the other was 200 μL of PBS alone to evaluate for autofluorescence.

To each tube containing 200 μL of the above monoclonal antibody or control, 100 μL of blood was added with gentle vortex mixing. The tubes were then incubated for 45 minutes at 4°C in darkness. The cells were washed twice with 3 μL of PBS centrifuging at 400 × g for three minutes at room temperature. After removal of the supernatant, 1 mL of immunofluorescence (Coulter) was added. The tubes were incubated for 90 s after which 250 μL of 10 percent formaldehyde in PBS was added. The cells were then washed with 2 μL of PBS followed by a 3-μL PBS wash, each time centrifuging at 400 × g for three minutes at room temperature. At this point, the cells were resuspended in 0.5 mL of PBS for analysis in the flow cytometer (Coulter Epics Clinical Flow Cytometer). This device was used to determine the relative proportions of lymphocytes labeling with CD3, CD4, CD8, and CD11 antigens.

Peripheral Blood: Human immunodeficiency virus cultures were performed by methods previously described. Briefly, 5 million peripheral blood lymphocyte (PBLs) were obtained by Ficoll-Hypaque centrifugation and cultured for four days with phytohemagglutinin (PHA). These cells were then cocultured with cells obtained three days after incubating PBLs from infected donors in the presence of PHA. Supernatants were collected at regular intervals for the next 17 days and treated with octoxynol-9 (Triton X-100) to inactivate any live virus. These were assayed for reverse transcriptase activity by testing for DNA oligonucleotide formation after addition of a tritiated nucleotide. Coculture cell growth was maintained by the addition of interleukin 2, and interferon produced by the lymphocytes was bound by adding sheep anti-interferon-alpha serum. A positive culture was defined as one in which reverse transcriptase activity was three times that in control cultures. For instances in which activity was between two and three times controls, supernatants were tested for the presence of viral antigen by P24 antigen capture enzyme-linked immunosorbent assay (ELISA). The presence of this antigen was considered confirmation of culture positivity.

BAL Fluid

The specimen was centrifuged at 400 × g for five minutes and the cell pellet was resuspended and cocultured with 5 million PBLs from uninfected donors for three days. Handling of the cultures for the next 17 days was identical with the peripheral blood assay given above.

Statistical Analysis

When applicable, data are expressed as mean ± 1 SD. Statistical significance of differences between various subgroups was determined by two-tailed unpaired t test or by analysis of variance.
RESULTS

A total of 41 HIV-seropositive WR stage 1 to 5 patients were enrolled in the study. Two patients were excluded (one because of blood contamination of the BAL fluid and another because of technical problems with processing the fluid), leaving 39 patients in the study group. Five of the 39 were anemic (hematocrit range, 33.9 percent to 39.3 percent). Eighteen (46 percent) of these patients complained of symptoms of dyspnea on exertion, including two of the anemic patients. None complained of new onset of cough or a change in cough pattern or fever. The WR stages of these patients are as follows: WR1, 13 patients; WR2, 4 patients; WR3, 4 patients; WR4, 6 patients; and WR5, 12 patients. Concurrently a control group of ten healthy HIV-seronegative subjects underwent BAL for comparison of cellular recovery. Also, during the study interval nine patients with AIDS (WR6) underwent BAL. Six of these had acute pulmonary infections (five had P carinii pneumonia and one had P carinii and cryptococcal pneumonitis). Table 1 shows the demographic characteristics of the patients and controls. Two of the WR1 to WR5 patients and one WR6 patient were taking zidovudine. None of the WR1 to WR5 patients was taking anti-Pneumocystis prophylaxis.

Of the 39 WR1 to WR5 patients, all but two had normal chest roentgenograms. These two patients, one a WR3 and the other a WR5, had a mild increase in interstitial markings. The former had mild gallium 67 uptake diffusely in the pulmonary parenchyma. Both had negative microbial stains and cultures of BAL fluid. An additional 33 BAL fluid samples were evaluated for infecting organisms (including all of the symptomatic patients). Examination of the fluid for organisms with AFB and GMS stains was negative in every case. There was no evidence of viral infection on Papanicolaou staining. Mycobacterial cultures were negative in all cases and fungal cultures were negative in all but five fluids (Aspergillus fumigatus grew in one, Candida species grew in three, and Penicillium species grew in one). Normal nasopharyngeal flora grew from bacterial cultures in nearly all of the fluids and there was light growth of Staphylococcus aureus in one case. Nocardia was not isolated from any fluid.

Twenty-three patients (WR1 to WR5) had HIV cultures of blood of which 14 (60.9 percent) were positive. Sixteen patients had BAL HIV cultures in addition to the blood cultures of which three (18.8 percent) were positive (two patients were WR1 and one was WR4). All three also had positive blood HIV cultures.

Table 2 shows BAL cellular recovery and differential cell counts. The WR1 to WR5 patients are subdivided into those with peripheral blood CD4 lymphocyte counts greater than 400/cu mm (WR1 and WR2) and those with less than 400/cu mm (WR3 to WR5). Similar data are shown in Table 3 with all smokers excluded. Similar amounts of fluid were instilled and recovered in all four groups. The overall cellularity was greater in the WR1 and WR2 patients than in the controls, but the statistical significance is lost when the smokers are excluded. There is a trend toward there being a greater percentage of lymphocytes and a lower percentage of macrophages in the WR6 patients vs the healthy controls that reaches statistical significance once the smokers are excluded. The WR1 to WR5 patients manifested similar differential cell counts as the controls with the exception of slightly more polymorphonuclear leukocytes and eosinophils in the WR3 to WR5 groups. Exclusion of the two patients with abnormal chest roentgenograms (both smokers) made no difference in any of these analyses.

Twenty-three BAL fluid samples were sent for lymphocyte subset analysis from among the WR1 to WR5 groups. None was sent from the control or WR6 groups. Twelve fluid samples were excluded from this analysis because of having too few lymphocytes for

Table 2—Bronchoalveolar Lavage (BAL) Cell Counts and Differentials

<table>
<thead>
<tr>
<th>Walter Reed Class</th>
<th>N</th>
<th>BAL Fluid Instilled, ml</th>
<th>BAL Fluid Recovery, ml</th>
<th>Cells Recovered per ml, × 10⁶</th>
<th>Percent*</th>
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<td></td>
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<td>Macro</td>
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<td>Controls</td>
<td>10</td>
<td>231 ± 63</td>
<td>134 ± 49</td>
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<td>77.0 ± 10.3</td>
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<td>22.7 ± 10.5</td>
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<td>1-2</td>
<td>17</td>
<td>247 ± 21</td>
<td>127 ± 22</td>
<td>4.4 ± 2.4†</td>
<td>79.3 ± 11.3</td>
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<td>0.4 ± 0.8</td>
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<td>3-5</td>
<td>22</td>
<td>232 ± 35</td>
<td>125 ± 9</td>
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<td>0.8 ± 0.9†</td>
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<td>6</td>
<td>9</td>
<td>230 ± 71</td>
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<td>0.1 ± 0.4</td>
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*Macro = macrophage; lymph = lymphocyte; PMN = polymorphonuclear leukocyte; and Eos = eosinophil.
†p < .05 compared with Walter Reed 0.
‡p < .02 compared with Walter Reed 0.
analysis or having too many cells labeling with monocyte markers in the lymphocyte gate. Eleven fluid samples remained for further analysis. Table 4 shows the comparison of BAL CD4 cell numbers and CD4/CD8 ratios between those with less than 400 CD4 cells in peripheral blood and those with this number or greater. There was no significant difference between the two groups.

**DISCUSSION**

The primary purpose of this study was to evaluate a population of HIV-seropositive patients for evidence of subclinical opportunistic infection. *Pneumocystis carinii* is the most common pulmonary pathogen in patients with AIDS. This organism was found in none of the 35 fluid specimens in which it was sought in the present study. This is striking in light of the complaints of dyspnea on exertion by many of the patients and peripheral blood CD4 counts less than 400/cu mm in 22 of the 39. Cytomegalovirus (CMV) infection is also common in patients with AIDS. Although CMV cultures were not obtained, no cytologic evidence of this organism was seen in any of the fluids. Cytologic study has been reported to have a negative predictive value of 38 to 92 percent for CMV pneumonia so that its presence cannot be conclusively excluded. Mycobacterial, fungal, and bacterial cultures and stains were also negative with the exception of the cultures listed above which most likely represent contaminates, saprophytes, or upper airway flora. These results imply that HIV-seropositive patients with normal chest roentgenograms are unlikely to harbor *P. carinii* or other pulmonary opportunistic organisms even when complaining of dyspnea on exertion. It is important to note that none of the 35 patients complained of a new cough or a change in cough pattern, fever, or dyspnea at rest—findings frequently seen in *P. carinii* infection. It is possible that these findings may be more specific for pulmonary infections than dyspnea on exertion is in the HIV-infected patient.

Two of our patients had increased interstitial markings on chest roentgenogram and one of these had mild pulmonary parenchymal gallium uptake. No pathogens were isolated in BAL fluid. Although biopsies were not done, these patients were thought to have one of the interstitial lung diseases described in HIV infection—lymphocytic interstitial pneumonitis or nonspecific interstitial pneumonitis although as noted above, CMV pneumonitis cannot be conclusively excluded.

Our findings are in keeping with those of Ognibene et al who studied 12 patients with AIDS diagnosed by nonpulmonary manifestations and another 12 HIV-seropositive patients with CD4 counts less than 200/cu mm. No patient with dyspnea, cough, sputum, fever, or abnormal chest roentgenogram was included. No evidence of *P. carinii* was found in any BAL fluid in this asymptomatic population. Taken together, these studies suggest that the finding of *P. carinii* on BAL should be considered as evidence of infection in the HIV-seropositive patient who has not previously had *P. carinii* pneumonitis. There does not appear to be a colonization phase identifiable by standard microbiologic staining techniques. Furthermore, an asymptomatic patient with a normal chest roentgenogram is unlikely to harbor pulmonary opportunistic pathogens. Likewise, an isolated complaint of dyspnea on exertion is unlikely to be explained by such infection.

The HIV cultures of BAL fluid revealed a similar positivity rate as has been noted in BAL fluids from AIDS patients in the past. The rate is considerably less than that obtained from culturing an equal volume of blood. Since HIV is associated with inflammatory
The BAL cell count and differential data are presented two ways—with all patients and with only the nonsmokers. This was done because all of the controls were nonsmokers and because of the variable affect of smoking on these measurements (primarily to increase cellularity and percentage of polymorphonuclear leukocytes and to lower the percentage of lymphocytes). All four groups had similar BAL fluid recovery. All three HIV-infected groups had greater cellularity than the controls did, but this did not reach statistical significance in any group once the smokers were eliminated. Like the AIDS patients studied by previous investigators, our WR6 patients had a higher percentage of lymphocytes and lower percentage of macrophages than the controls did. As in these previous studies, most of our AIDS patients had active pulmonary infections at the time they underwent BAL so that it is difficult to know whether the HIV infection or the opportunistic infection is responsible for the BAL fluid findings. Our own data suggest that the latter possibly is more likely given that the WR1 to WR5 patients had no difference in lymphocyte and macrophage percentages as compared with controls. However, one cannot exclude the possibility that the differential cell count changes once the lung finally loses its ability to defend against opportunistic pathogens such as P carinii but before the onset of a clinical infection. This question could probably best be answered by studying AIDS patients after recovery from a pulmonary opportunistic infection.

Our finding of similar macrophage and lymphocyte differentials in WR1 to WR5 patients is similar to the data of Agostini et al who noted no difference in BAL differentials between controls and 11 HIV-seropositive patients with constitutional or neurologic symptoms but not opportunistic infections. However, it is in contrast to the findings of Venet et al who studied 20 patients with chronic generalized adenopathy compared with 39 previously studied normal controls. Smokers were included in both groups. A significantly higher percentage of lymphocytes was noted in the patients (26.6 ± 22.6 percent vs 5 ± 4 percent). Likewise, Guillon et al noted a lymphocytosis (defined as >15 percent lymphocytes) in 20 of 36 HIV-seropositive patients without AIDS. The latter study was uncontrolled. The disparity between these studies and our own findings may be explained by our control group. The percentage of lymphocytes in BAL fluid from our controls is high by the standards of some laboratories, although not by others. These were done concurrently with our patients by the same technical personnel. Given the known difficulties distinguishing large lymphocytes from small macrophages in BAL fluid, such a control group is necessary to ensure that seemingly abnormal differential cell counts are not just due to bias on the part of the observer. Another possible explanation for the differences noted in our data compared with these previous studies is that their patients presented primarily because of symptoms and ours were identified by screening. Fifty-four percent were asymptomatic and the rest complained only of dyspnea on exertion. This population difference may result in a difference in the level of organ dysfunction in their population vs our own.

The lymphocyte subset analysis was limited just to the WR1 to WR5 patients. The CD4 and CD4/CD8 ratio in the fluid was similar between the WR1 and WR2 and WR3 to WR5 groups despite the large difference in peripheral blood CD4 and CD4/CD8 ratios between groups. This is not surprising given that CD4/CD8 ratios in BAL fluid have been found to correlate poorly with those in peripheral blood in other diseases such as sarcoidosis.

In conclusion, BAL fluid is unlikely to reveal opportunistic organisms in HIV-seropositive patients complaining of dyspnea on exertion but with normal chest roentgenograms. Such fluid does in some cases harbor the HIV organisms although culturing it is more difficult than it is from peripheral blood. The fluid contains inflammatory cells in similar ratios as normal subjects although perhaps in greater overall quantities. Further studies are necessary to delineate the functional decline in these cells as the lung loses its ability to defend itself against opportunistic organisms.

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