Alveolar Immune Mediators in HIV-Related Pneumonia*

Different Role of IL-2 and IL-1 in Inducing Lung Damage

Alberto Biglino, M.D.; Brunella Forno, M.Sc.; Anna M. Pollono, M.Sc.;
Paolo Ghio, M.D.; and Carlo Albera, M.D.

In order to elucidate the role played by alveolar cytokines in the pathogenesis of HIV-related lung damage, levels of interleukin (IL) 1 β, IL-2, IL-6, tumor necrosis factor (TNF)-α, and interferon (In) were assessed on supernatant of bronchoalveolar lavage fluid from 30 consecutive HIV-1 seropositive (HIVAb+) patients with clinical and radiologic evidence of pneumonia, from 20 HIV-seronegative (HIVAb−) patients with pulmonary sarcoidosis, and from 10 HIVAb− healthy control subjects. Cytokine levels were expressed as picogram (IL-1, TNF), nanogram (IL-6), and international unit (IL-2, In) per milligram of albumin per deciliter. Total and differential cell counts, cytofluorimetric enumeration of CD3+, CD3+/DR+, CD4+, CD8+, and CD8+/CD16+ cells, as well as microbiologic investigations for opportunistic agents were performed on lavage pellets. HIV-related pneumonia was characterized by higher mean alveolar level of IL-2 (12±5 IU) and by more elevated mean counts of T cells (109±16), activated T cells (60±12), and CD8+ cells (90±13)/μl if compared with both active sarcoidosis and control subjects, where respective values were 0.2±0.1 and 0.3±0.2 IU IL-2/mg Alb/dl, of 52±11 and 7±2 T cells, of 20±5 and 1.2±0.3 activated T cells, and of 11±2 and 3±0.6 CD8+ cells per microliter were found. HIV-infected patients with opportunistic lung infections (Ols) showed the highest mean IL-2 level (21±4 IU), and higher counts of both CD8+ (117±20) and CD8+/CD16+ (36±7) cells per microliter if compared with patients without evidence of Ols (respectively, 62±13 CD8+ and 18±3 CD8+/CD16+ cells per microliter). By contrast, extremely high IL-1 levels (1,463±760 pg), and IL-2 levels similar to control subjects (3.4±1.2 IU), were found in the absence of Ols. Different mechanisms depending respectively on IL-2-mediated cytotoxic cell recruitment and activation, or IL-1-mediated tissue injury may account for HIV-related lung damage, depending on the presence or absence of opportunistic agents. (Chest 1993; 103:439-43)

Infection by human immunodeficiency virus type 1 (HIV-1) is frequently associated with inflammatory lung involvement of both infectious and noninfectious origin. A lymphocytic alveolitis with prevalence of CD8+ cells is frequently reported in these patients even in the absence of opportunistic infections (OIs), as it is observed in nonspecific interstitial pneumonia (NIP) and in lymphocytic interstitial pneumonia (LIP), which account for 12 to 22 percent of HIV-related lung diseases, respectively, in adult and pediatric patients. Although the mechanisms responsible for lung damage in HIV infection remain obscure, a cytokine-mediated proliferation and/or recruitment of CD8+ cells from peripheral blood has been hypothesized. In fact, HIV-infected peripheral monocytes can release in vitro abnormal amounts of interleukin (IL)-1 and tumor necrosis factor (TNF), whose capacity of inducing both direct tissue injury and T-lymphocyte activation is well known. Production of TNF by isolated alveolar macrophages of HIV-infected patients has been reported. However, in vitro functional studies on bronchoalveolar lavage (BAL)-isolated cells may be influenced by the disruption of the alveolar environment. A study evaluating cytokine levels directly in the alveolar fluid, as an expression of local immunoregulation, seems therefore worthwhile. Among immune mediators, IL-1β, IL-2, IL-6, TNF-α, and interferon (In) are very likely to be involved in recruitment, activation, and expansion of mononuclear cells within the lung. For these reasons, we evaluated the levels of the above cytokines in bronchoalveolar lavage fluid (BALF) of a group of HIV-infected patients with clinical and radiologic evidence of pneumonitis; results were compared with those obtained from a group of patients with active, untreated sarcoidosis, and from a control group of subjects with minor hemoptysis of extrapulmonary origin. Results were correlated with bronchoalveolar cytogram, with alveolar lymphocyte phenotype, and with

*From the Istituto di Malattie Infettive (Drs. Biglino, Forno, and Pollono) and the Clinica di Malattie dell’Apparato Respiratorio (Drs. Albera and Ghio), University of Torino, Italy. This work was supported by a grant from the Fondazione Denegri, Torino, Italy. Manuscript received January 17; revision accepted June 19. Reprint requests: Dr. Biglino, Ospedale Amedeo di Savoia, Corso Solvizzera 164, 10149 Torino, Italy
the presence or absence of OIs.

**Materials and Methods**

**Study Design**

**Patients:** The study was carried out on a group of 30 consecutive subjects (26 men, 4 women; mean age, 34 ± 8 years; 24 smokers; 6 nonsmokers) attending our outpatient care facility because of HIV-1 infection with evidence of respiratory involvement. Inclusion criteria were as follows: clinical—fever >38°C since at least 2 weeks; cough and dyspnea on exertion; evidence of lung consolidation or crackles; radiological—evidence of interstitial and/or acinar involvement of lung parenchyma on chest roentgenogram performed within 48 h of clinical examination; and laboratory—presence of anti-HIV-1 serum antibodies, detected by two different commercial enzyme immunoassays and confirmed by Western blot analysis.

Exclusion criteria were as follows: age younger than 18 years or older than 50 years; presence of extrapulmonary opportunistic infections or HIV-related neoplastic manifestations; ongoing or previous antiviral or antineoplastic treatment; and arterial oxygen tension <50 mm Hg.

The results obtained from this population were compared with those obtained from 20 consecutive HIV-seronegative patients (9 men; 11 women; mean age, 40 ± 11 years; 13 smokers, 7 nonsmokers) with untreated, biopsy specimen-proven active pulmonary sarcoidosis who underwent BAL at a thoracic disease facility (Clinica di Malattie dell’Apparato Respiratorio, University of Torino) as part of routine study before starting steroid treatment.

**Control Population:** Ten healthy, HIV-seronegative subjects (seven smokers; 3 nonsmokers) presenting with minor hemoptysis of extrapulmonary origin (generally from upper airways) and with normal chest roentgenograms underwent bronchoevacuation and BAL during diagnostic workup 1 week to 10 days after bleeding had stopped.

**Bronchoalveolar Lavage**

A BAL was performed in all subjects through fibroscopes (Olympus BF 20) by instillation of 180 ml of sterile saline solution (0.9 percent) in three 60-ml aliquots. Lavage was carried out on middle or right inferior lobe in patients with diffuse infiltrates and in control subjects, and on the involved lobe in patients with localized radiologic abnormalities.

Recovered fluid was filtered through a single pad of sterile gauze to remove mucus and debris, and centrifuged at 400g. +4°C for 10 min. Clear supernatant was immediately frozen at −80°C, while cellular pellet was resuspended in sterile phosphate-buffered saline solution and employed for the following investigations.

**BAL Cytology and Microbiologic Investigations**

Total and differential cell counts were performed respectively on a hemocytometer (Burker) and on Giemsa-stained cytocentrifuge preparations of lavage pellet (Cytospin, Shandon Southern). Other Cytospin preparations were stained with Grocott, Zielh, periodic acid-Schiff (PAS), and Gram to detect protozoa, mycobacteria, fungi, and bacteria. Appropriate cultures to identify Herpetoviridae, as well as Mycobacteria, fungi, and bacteria were carried out on lavage pellets. More in detail, cytomegalovirus infection was diagnosed by immunofluorescent detection of early nuclear antigens on monolayers of MRC-5 cells; this technique combines a relative rapidity (24 h) with a sensitivity comparable to that of immunocytochemical methods, being free of false-positive results.13,14

**BAL Cells Phenotypic Characterization**

Aliquots of cellular pellets, suspended in phosphate-buffered saline solution containing 1 percent formaldehyde at a concentration of 1 × 10⁶ cells per milliliter were incubated for 30 min at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-Leu4 (CD3), anti-Leu3a (CD4), and anti-Leu2a (CD8) monoclonal antibodies (MoAbs). Double stainings were performed on different aliquots with FITC-conjugated anti-HLA-DR plus phycoerythrin (PE)-conjugated anti-Leu4, and with FITC-conjugated anti-Leu2a plus PE-conjugated anti-Leu 5a (Becton Dickinson), recognizing respectively CD3+/HLA-DR+ cells and CD8+/CD16+ cells. The percentage of cells positive for the above antibodies was determined by flow cytometry on an analyzer (FACSCAN, Becton Dickinson), and used to calculate absolute counts per microliter of, respectively, T cells, helper/inducer cells, suppressor/cytotoxic cells, activated T cells, and HLA-restricted cytotoxic T lymphocytes in native BALF.

**BALF Cytokine Assays**

Cytokine levels were determined on BALF supernatants, concentrated 20 × (+4°C) immediately after thawing with centrifuge devices (Centriprep, Amicon). Levels of IL-1β, IL-6, and TNF-α were assessed by immunoassays (IL-1: Cistron Biotec,ology, Pine Brook, NJ; IL-6, TNF-α: Genzyme Corporation, Boston).

Interferon and IL-2 were titrated on serial twofold dilutions of concentrated supernatants, respectively, with a cytopathogenic effect reduction assay on WISH cells, employing vesicular stomatitis virus as challenge,15 and with a stimulation test on mouse IL-2-dependent T cells (CTLL).16 Titeres were expressed as international units. Interferon type was characterized by repeating the bioassay after a 2-h incubation at 37°C with anti-human IFN-α and anti-human IFN-γ gamma monoclonal antibodies (Boehringer Mannheim, Germany). Albumin levels were determined on the same specimens by laser nephelometry; cytokine levels were expressed as picograms (IL-1β, TNF-α), nanograms (IL-6), or international units (IL-2, IFN) per milligram of albumin per deciliter of native BALF.

**Statistical Evaluation**

Results were expressed as mean values (geometric means for IFN and IL-2 titer) ± SEM. The significance of the observed differences between healthy subjects, patients with sarcoidosis, and HIV+ patients was assessed with analysis of variance (ANOVA) for cell counts, and with nonparametric Kruskal-Wallis test for cytokine levels. The significance of differences observed between HIV+ patients with and without OIs was assessed with Student's t test for

| Table 1—Phenotypic Analysis of BAL Cells From Normal (NORM) Subjects, Patients With Sarcoidosis (SARC), HIV-Infected Patients, HIV Patients With Opportunistic Infections (OI+), and HIV Patients Without Opportunistic Infections (OI−)* |
|----------------------|----------------------|----------------------|----------------------|----------------------|
| Cells/μl | NORM | SARC | HIV (Total) | p (ANOVA) | HIV (OI−) | HIV (OI+) | p (t Test) |
| Lymphocytes | 8 ± 2 | 57 ± 12 | 130 ± 19 | <0.0001 | 93 ± 16 | 164 ± 32 | 0.05 |
| CD3+ | 7 ± 1.8 | 52 ± 11 | 109 ± 16 | 0.0001 | 77 ± 15 | 141 ± 27 | 0.05 |
| CD3+/DR+ | 1.2 ± 0.3 | 20 ± 5 | 60 ± 12 | 0.0003 | 43 ± 13 | 76 ± 20 | 0.18 |
| CD4+ | 4.1 ± 0.8 | 41 ± 9 | 10 ± 3 | 0.0012 | 8 ± 2 | 11 ± 4 | 0.57 |
| CD8+ | 3 ± 0.6 | 11 ± 2 | 90 ± 13 | <0.0001 | 62 ± 13 | 117 ± 20 | 0.03 |

*Results are reported as mean cell counts per microliter of native BALF.

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Table 2—Mean BALF levels of IL-1 \(\beta\), TNF- \(\alpha\) (pg/mg Alb/dl), IL-6 (ng/mg Alb/dl), IL-2, and IFN (IU/mg Alb/dl) in Normal Subjects (NORM), Patients With Sarcoidosis (SARC), HIV-Infected Patients (HIV), HIV Patients Without Opportunistic Infections (OI-), and HIV Patients With Opportunistic Infections (OI+)

<table>
<thead>
<tr>
<th></th>
<th>NORM</th>
<th>SARC</th>
<th>HIV (Total)</th>
<th>HIV (OI-)</th>
<th>HIV (OI+)</th>
<th>(p) (Mann-Whitney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 (\beta)</td>
<td>32±11</td>
<td>21±8</td>
<td>709±430</td>
<td>0.55</td>
<td>1463±760</td>
<td>35±22</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.3±0.2</td>
<td>0.2±0.1</td>
<td>12±5</td>
<td>0.0008</td>
<td>3.4±1.2</td>
<td>21±4</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.36±0.3</td>
<td>0</td>
<td>1.9±0.8</td>
<td>0.0024</td>
<td>2.5±1.5</td>
<td>1.2±0.6</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>175±54</td>
<td>207±67</td>
<td>292±118</td>
<td>0.444</td>
<td>108±60</td>
<td>463±189</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>1.1±0.9</td>
<td>2.9±1.2</td>
<td>9±2.8</td>
<td>0.104</td>
<td>11±4</td>
<td>7±3</td>
</tr>
</tbody>
</table>

**Results**

HIV-infected patients with pneumonia had evidence of a lymphocytic alveolitis characterized by significantly higher counts of T cells, activated T cells, and suppressor/cytotoxic cells per microliter (Table 1), and by significantly higher mean IL-2 and IL-6 levels (Table 2) if compared both to patients with active sarcoidosis and to control subjects. Mean IL-1 and IFN-gamma levels, though strikingly elevated in HIV+ patients, did not differ significantly from those observed in sarcoidosis patients or in the control group. T-helper/inducer cells were present in HIV+ patients' BAL in amounts comparable to those observed in normal subjects, albeit significantly lower than those observed in patients with sarcoidosis (Table 1).

In 15 HIV+ patients, an OI was evidenced by analysis of BAL (Pneumocystis carinii in six cases, cytomegalovirus in six cases, C neoformans in three cases), while in the remaining patients OIs were ruled out.

HIV+ patients with opportunistic lung infections had significantly higher counts of total alveolar lymphocytes, T cells, and CD8+ cells per microliter if compared to patients without opportunistic infections (Table 1); there were no significant differences concerning CD4+ cells both at the alveolar level (Table 1), as well as in peripheral blood, where mean counts of 93±29 and 186±58 CD4+ cells per microliter (\(t = 1.44\); \(p = 0.185\)) were observed, respectively, in patients with and without OIs. HLA-restricted cytotoxic T cells (CD8+/CD16+ \(\beta\)) were also present in a significantly higher amount in BAL from HIV+ patients with opportunistic infections (36±7/\(\mu\)l) if compared to patients with pneumonia unrelated to opportunistic agents (18.2±3/\(\mu\)l; Fig 1).

HIV-infected patients with opportunistic lung infections showed significantly higher levels of IL-2, while in patients with pneumonia unrelated to OIs, significantly higher levels of IL-1 were evidenced (Table 2). A positive linear correlation (\(r = 0.47\)) was evidenced between BALF IL-2 levels and CD8+/CD16+ cell counts per microliter (Fig 2). No other significant correlation could be found between alveolar cytokine levels and cell type involved in alveolitis (Table 3). Finally, no significant differences were found between all groups as far as TNF levels are concerned.
Table 3—Linear Correlations ("R" Coefficients) Between Levels of Alveolar Immune Mediators and Absolute Lymphocyte Counts in HIV+ Patients

<table>
<thead>
<tr>
<th></th>
<th>IL-1</th>
<th>IL-2</th>
<th>IL-6</th>
<th>TNF</th>
<th>IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>-0.20</td>
<td>0.06</td>
<td>-0.23</td>
<td>-0.05</td>
<td>-0.16</td>
</tr>
<tr>
<td>CD3+</td>
<td>-0.21</td>
<td>0.10</td>
<td>-0.24</td>
<td>0.01</td>
<td>-0.14</td>
</tr>
<tr>
<td>CD3+/DR</td>
<td>-0.10</td>
<td>0.14</td>
<td>-0.19</td>
<td>0.11</td>
<td>-0.08</td>
</tr>
<tr>
<td>CD4+</td>
<td>-0.18</td>
<td>0.04</td>
<td>-0.21</td>
<td>-0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>CD8+</td>
<td>-0.21</td>
<td>0.14</td>
<td>-0.26</td>
<td>0.04</td>
<td>-0.15</td>
</tr>
<tr>
<td>CD8+ /CD16</td>
<td>-0.30</td>
<td>0.47</td>
<td>-0.11</td>
<td>0.28</td>
<td>-0.18</td>
</tr>
</tbody>
</table>

*F = 6.86; p = 0.015.

Discussion

In this study we confirmed that HIV-related pneumonia is characterized by a lymphocytic alveolitis with higher CD3+, CD3+/HLA-DR+, and CD8+ cell counts if compared to the alveolar cell phenotype of sarcoidosis, where a CD4+ cell alveolitis predominates. In our patients, however, alveolar CD4+ cell mean count, though significantly lower than that observed in sarcoidosis, was similar to that observed by us (and already reported by others) in healthy control subjects. Furthermore, both alveolar and peripheral blood mean CD4+ counts were independent of the presence or absence of opportunistic lung infections. These observations suggest that HIV-induced CD4+ cell loss is not a fundamental requisite for the development of pneumonia in HIV infection, and that either a recruitment or a local expansion of cytotoxic cells is involved in HIV-related lung damage. The mechanism may be similar to that hypothesized to explain CD4+ cell alveolitis in sarcoidosis, but it is likely to involve a different cell subset because of a different initial event. Both macrophage- and lymphocyte-derived mediators of immunity are among the most powerful factors conditioning lymphocyte recruitment to the lung, as already demonstrated in sarcoidosis12,18 and in hypersensitivity pneumonitis.19 Local dysregulation of cytokine system, possibly induced by HIV itself, may play a key role in the pathogenesis of HIV-related lung disease.

This hypothesis was supported by the extremely high IL-1-1 levels we found in the absence of OIs, and, in their presence, by significantly higher levels of IL-2 if compared not only to control population, but even to sarcoidosis patients. The low IL-2 levels found by us in sarcoidosis patients' BALF, in spite of a lymphocytic alveolitis with prevalence of CD4+ cells, is only in partial contrast to previous articles reporting a spontaneous IL-2 release from alveolar lymphocytes in vitro. In the lung, the same unknown event responsible for CD4+ cell recruitment, activation, and proliferation may cause in these cells a "post-activation" state making them unable to further release IL-2 spontaneously.20 The opposite seems to happen in HIV-related pneumonia, where macrophage infection by HIV, though not lethal for the cell, may elicit important derangements of cytokine release, mainly consisting of uncontrolled release of both IL-1 and TNF.21 IL-1 produced in situ powerfully activates alveolar wall endothelial cells, enhancing adherence and entry of circulating, either antigen-sensitized or nonspecific lymphocytes.22 In patients without evidence of OIs, lung damage could be due either to the direct toxic effect of IL-1 β (released by HIV-infected macrophages) on alveolar vascular endothelial cells or to IL-1-induced, nonspecific compartmentalization of cytotoxic T cells.24 The first hypothesis considering a direct damage seems more in accordance with our results, as no positive correlation could be found by us between alveolar IL-1 β levels and either total lymphocyte counts or T cell subpopulations. HIV infection per se may be the only explanation of alveolitis in these cases. The opposite was evident in the case of pneumonia due to opportunistic infections, where almost no alveolar IL-1 was evidenced, but the highest levels of alveolar IL-2 and of activated T cells, CD8+ cells, and HLA-restricted cytotoxic T cells (CD8+/CD16+) were detected. An IL-2-induced local proliferation of cytotoxic T cells leading to immunemediated lung damage could be hypothesized in this situation. The presence of such high levels of IL-2 was not impossible, since CD4+ cells were detectable in amounts similar to those observed in normal subjects, though lower than those detected in patients with active sarcoidosis. Furthermore, CD4+ cell number does not seem to be critical for IL-2 production, as these cells may retain IL-2-producing ability independently of a reduction of proliferating activity, as already demonstrated in patients with sarcoidosis and in normal subjects.30 As a consequence of macrophage saturation by opportunistic agents, a defective IL-1 release coupled with nonlethal viral injury to CD4+ cells could be responsible for a lack of proliferation of helper cells, which retain the capacity of producing IL-2. This may lead to uncontrolled proliferation of CD8+ cytotoxic cells already recruited to the lung as a consequence of HIV infection. This observation is supported by the high number of T-activated (CD3+/DR+) as well as of HLA-restricted cytotoxic (CD8+/CD16+) alveolar cells, and by the positive linear correlation was found between IL-2 levels and the number of CD8+/CD16+ in opportunistic-related pneumonia. Moreover, the almost undetectable IL-1 levels we observed in BALF of patients with OIs are in accordance with a functional and/or numerical defect of alveolar macrophages representing the main target of both opportunistic agents and of cytotoxic cell-mediated injury.

In conclusion, different mechanisms involving local lymphokine derangements seem to be responsible for HIV-related lung damage, depending on the presence

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or absence of OIs. Further investigations are needed to elucidate the relationships between the extent of alveolar cell infection (HIV-1 BAL titer, p24 antigen level), and both phenotypical and functional characteristics of alveolar macrophage.

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