Phenotypes and Lymphokine-Activated Killer Activity of Pleural Cavity Lymphocytes of Lung Cancer Patients Without Malignant Effusion*

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We examined the phenotypes of lymphocytes in the pleural cavity of 23 lung cancer patients without malignant effusion. The ability of those lymphocytes to develop lymphokine-activated killer (LAK) activity and the regulation of LAK by pleural cavity macrophages were also compared with their counterparts in the peripheral blood. Mononuclear cells (MNC) were obtained simultaneously from the blood and by lavage of the pleural cavity of patients with lung cancer. The proportion of the T-cell subset of HLA-DR+ cells was significantly higher in the pleural cavity than in the peripheral blood, but the proportions of CD3+ and CD8+ cells in the pleural cavity were similar to the corresponding proportions in the blood. The proportions of CD4+ and CD16+ cells were lower in the pleural cavity than in the blood. The LAK activity could be developed by MNC from the pleural cavity following incubation with interleukin 2 (IL-2), but the LAK activity of pleural cavity MNC was significantly less than that of peripheral MNC. Pleural cavity lymphocytes alone also developed LAK activity following incubation with IL-2. Pleural macrophages from the patients were regulated to augment in vitro induction of LAK activity by IL-2 from autologous blood lymphocytes and pleural cavity lymphocytes. Lymphocytes in the pleural cavity without malignant pleural effusion could be developed by LAK activity and this activity was augmented by pleural cavity macrophages. The LAK activity developed by pleural cavity lymphocytes was significantly lower than that developed by peripheral blood lymphocytes. However, they can change their population to include cells with higher activities on exposure to IL-2 against the invasion of lung cancer cells into the pleural cavity. Thus, the population of lymphocytes in the pleural cavity of patients with lung cancer without malignant pleural effusion was different from that in malignant pleural effusion.

(Chest 1993; 103:1732-36)

Malignant pleuritis is frequently observed in association with malignant neoplasms of the lung.1-6 Several groups previously have studied the characteristics of malignant effusion. Kay et al7 have reported reduced levels of IgG in malignant pleural effusion. Others have observed increased levels of immune complexes in malignant effusions.8,9 Phenotypically, CD4+ cells are significantly more common in malignant effusion than in peripheral blood.10-12 Natural killer (NK) activity was found to be lower in malignant pleural effusion than in peripheral blood.13 Unprimed human lymphocytes cultured in vitro with interleukin 2 (IL-2) for four to six days without antigenic stimulation develop nonspecific antitumor cells that can destroy various fresh autologous and allogeneic tumor cells14,15 and are named as lymphokine-activated killer (LAK) cells.16-18 The development of LAK activity has been found to be upregulated or downregulated by healthy human monocyte-macrophages.19-22 In certain cancer patients, impaired activity of LAK cells and a suppressor factor have been demonstrated.23,24

In patients with lung cancer, invasion of cancer cells into the pleura and/or pleural cavity might be influenced by both the nature of the cancer cells and host factors.25 Recently, we found that most lymphocytes present in malignant pleural effusion are CD4+ cells.26 We found, in addition, that lymphocytes in malignant pleural effusion could respond to IL-2 to the same extent as could blood lymphocytes in the development of LAK activity, and that LAK development was upregulated by macrophages in malignant pleural effusion.27

Little is known, however, about the biologic activities of various cells in the pleural cavity before its invasion by cancer cells. We, therefore, examined the phenotypes of pleural cavity lymphocytes and the ability of these lymphocytes to develop LAK activity, and also the regulation of LAK by pleural cavity macrophages in lung cancer patients without malignant pleural effusion.
**Materials and Methods**

**Patients**

Studies were made of 23 patients with resectable primary lung cancer not associated with malignant pleural effusion. None of the patients had received any anticancer therapy prior to the study. Computed tomography and magnetic resonance imaging were used to identify whether pleural effusions were present. The clinical characteristics of the patients are summarized in Table 1. Histologically, the cancers included 12 squamous cell carcinomas, 7 adenocarcinomas, and 4 large cell carcinomas. The TNM classification system (Union Internationale Contre la Cancer, 1987) was used for staging of the disease. Six patients were classified as having stage I, one as having stage II, 10 as having stage IIIA, one as having stage IIIB, and 5 as having stage IV. Four patients classified as having stage IV had pulmonary metastasis in the same lobe and one had superior vena cava syndrome and adrenal gland metastasis but they had no malignant pleural effusions. Eight patients had pleural invasion extending beyond the visceral pleura to the neighboring lobe or chest wall pathologically but no associated pleural effusion. The invaded neighboring lobe or chest wall was also removed. At the time of surgery, it was confirmed that no malignant pleural effusion was present finally.

**Reagents**

Petal bovine serum (FBS) was purchased (M.A. Bioproducts, Walkersville, Md). Recombinant human IL-2 (Takeda Pharmaceutical Co, Osaka, Japan), had a specific activity of 3.5 × 10^6 U/mg as assayed on IL-2-dependent murine NKC3 cells.**

**Cell Culture**

Human Burkitt lymphoma cells (Daudi) (American Type Culture Collection, Rockville, Md) were maintained as stationary suspension cultures in RPMI 1640 medium supplemented with 10 percent heat-inactivated FBS and gentamicin, designated as CRPMI 1640, at 37°C in a humidified atmosphere of 5 percent CO₂ in air.

**Isolation and Culture of Pleural Cavity Macrophages and Peripheral Blood Monocytes**

Pleural lavage was performed as follows.** At thoracotomy, it was confirmed that no malignant pleural effusion was present in the pleural cavity. The pleural cavity was then washed with 1,000 ml of 0.9 percent NaCl solution (saline solution) at 37°C. The washing fluid was collected aseptically in heparinized (10 U/ml) centrifuging bottles and centrifuged at 1,200 rpm (400 × g) for 10 min. The cell pellets were resuspended in 15 ml of phosphate-buffered saline solution. At the same time, 20 ml of peripheral blood was taken from each patient in a heparinized syringe to obtain samples of peripheral blood lymphocytes and monocytes. Mononuclear cells (MNCs) containing pleural cavity macrophages or peripheral blood monocytes were separated from the lavage fluid and from the peripheral blood by discontinuous gradient centrifugation in lymphocyte separation medium.** Then, as a further check, the absence of malignant cells among washed MNCs was confirmed morphologically by hematoxylin-eosin staining. Mononuclear cells from the pleural cavity and blood were tested for ability to induce LAK activity. The viability of these MNCs was more than 95 percent, as judged by the trypan blue exclusion test. Pleural cavity macrophages, peripheral blood monocytes, pleural cavity lymphocytes, and peripheral blood lymphocytes were isolated from the MNCs by discontinuous gradient centrifugation in 46 percent solution (Percol) at 1,800 rpm (600 × g) for 30 min. Next, the pleural cavity macrophage and peripheral blood monocyte suspensions were washed twice with RPMI 1640. More than 90 percent of the cells were either pleural cavity macrophages or peripheral blood monocytes, as judged by their morphologic features and staining for nonspecific esterase. Samples of 4 × 10⁶ pleural cavity macrophages or peripheral blood monocytes were plated in wells of 96-well plates (Microtest III plates, Falcon Plastics Co) and incubated for 1 h at 37°C. The monolayers of pleural cavity macrophages or peripheral blood monocytes were then washed twice with CRPMI 1640 to remove all nonadherent cells. At this point, the purity of the monocytes or macrophages was greater than 98 percent, as judged by their morphologic features and nonspecific esterase staining.

**Flow Cytometry**

A direct immunofluorescent assay was used to detect surface markers of pleural cavity blood lymphocytes. Monoclonal antibodies conjugated with fluorescein isothiocyanate (OKT monoclonal antibodies, Ortho Diagnostics, or Leu monoclonal antibodies, Becton-Dickinson) were added to the cell suspension, and the mixtures were incubated for 30 min at 4°C. The cells were then washed, resuspended in 0.1 ml phosphate-buffered saline solution, and analyzed (FACScan). The monoclonal antibodies used were those directed against lymphocyte-antigens CD3, CD4, CD8, CD16, CD25, CD57, and class II HLA-DR.

**LAK Activity Assay**

The LAK activity was assayed by measuring ⁴⁰Cr release using a method described in detail previously.** Briefly, to induce LAK activity, precursor cells (lymphocytes from the pleural cavity or blood) with or without monocytes/macrophages at a lymphocyte/macroage ratio of 10:4 were incubated with IL-2 for four days in RPMI 1640 medium supplemented with 5 percent FBS and gentamicin at 37°C under 5 percent CO₂ in humidified air. Unless otherwise noted, 400 U/ml of IL-2 was used for induction of LAK activity in MNCs from lavaged pleural fluid or blood; we have found that this concentration of IL-2 is optimal for induction of maximal LAK activity from blood lymphocytes.** There was no significant difference between the numbers of cells after four days of culture with and without the presence of IL-2. The cytotoxicities of these cultured cells and freshly isolated lavaged fluid of the pleural cavity and peripheral blood lymphocytes against ⁴⁰Cr-labeled Daudi cells (10⁶) were measured at 10:1 for triplicate cultures. Also, the cytotoxicities of freshly isolated lavaged fluid of the pleural cavity and peripheral blood MNCs against ⁴⁰Cr-labeled Daudi cells (10⁶) were measured at 20:1, 10:1, 5:1 effector/target ratios for triplicate cultures. Coculture of effector cells and target cells were terminated after 4 h, and the radio activities of the supernatants (0.1 ml per well), separated by brief centrifugation at 05 × g, were determined.

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**Table 1 — Clinical Features of Patients**

<table>
<thead>
<tr>
<th>Features</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>23</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
</tr>
<tr>
<td>Age, yr</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>66</td>
</tr>
<tr>
<td>Range</td>
<td>46-78</td>
</tr>
<tr>
<td>Type of lung cancer</td>
<td></td>
</tr>
<tr>
<td>Squamous cell</td>
<td>12</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>7</td>
</tr>
<tr>
<td>Large cell</td>
<td>4</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
</tr>
<tr>
<td>IIIA</td>
<td>10</td>
</tr>
<tr>
<td>IIIB</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
</tr>
</tbody>
</table>
Using a gamma counter, the percentage of cytotoxicity was calculated as follows:

\[
\text{% cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100
\]

The spontaneous release was about 10 percent (range, 7 to 13 percent) of that on total lysis. One lytic unit (LU) was defined as the number of effector cells required for 80 percent lysis of target cells.*

**Statistical Analysis**

The statistical significance of differences between values obtained for test groups was determined by using the paired t test.

**Results**

**Phenotypic Characterization of Lymphocytes in the Pleural Cavity**

In the present study, we examined the phenotypes of lymphocytes in the peripheral blood and pleural cavity of ten patients with lung cancer without malignant effusion. As shown in Figure 1, these patients had ranges of 41.1 to 83.3 percent CD3* cells (mean, 64.6 percent), 32.7 to 61.0 percent CD4* cells (mean, 47.6 percent), 9.3 to 42.0 percent CD8* cells (mean, 22.7 percent), 6.0 to 27.6 percent CD16* cells (mean, 18.6 percent), 1.4 to 4.9 percent CD25* cells (mean, 3.1 percent), and 15.7 to 34.8 percent HLA-DR* cells (mean, 23.7 percent) for peripheral blood lymphocytes, and 28.7 to 86.5 percent CD3* cells (mean, 59.9 percent), 11.5 to 57.7 percent CD4* cells (mean, 30.6 percent), 3.8 to 52.2 percent CD8* cells (mean, 27.7 percent), <1.0 to 10.7 percent CD16* cells (mean, 4.6 percent), <1.0 to 9.7 percent CD25* cells (mean, 3.2 percent), and 23.9 to 81.3 percent HLA-DR* cells (mean, 56.2 percent) for pleural cavity lymphocytes. The proportions of the CD4* T-cell subset and of the NK cell subset of CD16* cells were significantly higher in the peripheral blood than in the pleural cavity. On the other hand, there were more HLA-DR-positive lymphocytes in the pleural cavity than in the peripheral blood.

**In Vitro Induction of LAK Activity From MNCs in the Pleural Cavity**

Mononuclear cells from the blood and pleural cavity of the 13 patients examined were cytotoxic to Daudi cells when incubated for four days with IL-2. Significant LAK activity was induced in the blood MNCs (45±39 LU) and pleural cavity MNCs (26±27 LU) (mean±SD) of almost all patients when the MNCs were incubated with IL-2. Mononuclear cells from the blood of three patients (patients 2, 9, and 13) had spontaneous LAK activity even in the absence of IL-2 stimulation (8 to 20 LU). Also, all of their LAK activities could be augmented by IL-2 stimulation (55 to 105 LU). Mononuclear cells from the pleural cavity of two of them (patients 9 and 13) also had spontaneous LAK activities. The LAK activities of pleural cavity MNCs were significantly lower than those of blood MNCs (p<0.05) (Table 2).

**Table 2—LAK-Mediated Lysis of Tumor Cells by MNC From Peripheral Blood and the Pleural Cavity (LU/10⁶ Cells)**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Peripheral Blood</th>
<th>Pleural Cavity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2 (−)</td>
<td>IL-2 (+)</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>67</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>&lt;1</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>&lt;1</td>
<td>109</td>
</tr>
<tr>
<td>7</td>
<td>&lt;1</td>
<td>98</td>
</tr>
<tr>
<td>8</td>
<td>&lt;1</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>10</td>
<td>&lt;1</td>
<td>31</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
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</tr>
<tr>
<td>12</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>13</td>
<td>20</td>
<td>105</td>
</tr>
</tbody>
</table>

*LAK = lymphokine-activated killer; MNC = mononuclear cells; LU = lytic unit; IL-2 = interleukin 2. Freshly isolated mononuclear cells were cultured for four days in the presence or absence of 400 U/ml of IL-2 in 96-well plates before assays.

Pleural Cavity Lymphocytes of Lung Cancer Patients (Takahashi et al)
Upregulation of LAK Induction by Macrophages From the Pleural Cavity

As shown in Figure 2, blood lymphocytes were observed to have LAK activity after IL-2 stimulation, whereas blood monocytes or macrophages from the pleural cavity alone were not cytotoxic to Daudi cells. The LAK activities of blood lymphocytes were, however, significantly increased by the addition of blood monocytes. Similarly, the blood lymphocytes of all the patients were observed to have increased LAK activities in the presence of macrophages from the pleural cavity at a lymphocyte/macrophage ratio of 10:4.

The LAK activities of lymphocytes from the pleural cavity were also significantly increased by the addition of blood monocytes (Figure 3). Similarly, the LAK activities of pleural cavity lymphocytes from six of seven patients tested increased when stimulated with IL-2 in the presence of pleural cavity macrophages.

DISCUSSION

In this study, we examined the phenotypes of lymphocytes in the pleural cavity of lung cancer patients without malignant effusion. The ability of those lymphocytes to develop LAK activity and the regulation of LAK by pleural cavity macrophages were also compared with their counterparts in the peripheral blood.

There have been many studies of malignant pleural effusions. Domagala et al. reported that the percentages of T cells in pleural effusions of patients with neoplastic disease were higher than those in peripheral blood. Other investigators have reported that the populations of CD3+ cells and CD8+ cells in malignant pleural effusions are similar to those in peripheral blood. The proportion of CD3+ cells and CD8+ cells in the pleural cavity lavage fluid without malignant cells were also found to be similar to those of peripheral blood. On the other hand, the proportion of CD4+ cells was found to be significantly higher in malignant pleural effusions than in peripheral blood. Recently, we confirmed the result, finding that the population of cells in malignant pleural effusions due to lung cancer contained approximately 79 percent CD3+ cells, 64 percent CD4+ cells, and 15 percent CD8+ cells. In the present study, the proportion of CD4+ cells in the pleural cavity without malignant effusion was found to be significantly less than that in the peripheral blood. This result, therefore, suggests that T lymphocytes in the pleural cavity may react to the invasion of lung cancer cells into the pleural cavity. CD4+ cells accumulated in malignant pleural effusions. In the pleural cavity, CD4+ cells probably play important roles in opposing the development of invasion of lung cancer cells. Miller reported that CD4+ cells predominantly produced IL-2, and so CD4+ cells might selectively accumulate into the pleural cavity as the source of IL-2. However, the activity of IL-2 produced by CD4+ cells might be blocked by soluble IL-2 receptors. Therefore, whether the increased CD4+ cells in malignant pleural effusions of lung cancer patients actually provide immunologic defense is still an important issue.
We also studied the ability of pleural cavity lymphocytes to develop LAK activity and the regulation of LAK by pleural cavity macrophages. The LAK activity of pleural cavity lymphocytes could be developed by incubation with IL-2 for four days. Mononuclear cells from the peripheral blood of three patients had spontaneous LAK activities even in the absence of IL-2 stimulation. Also, all of their LAK activities could be augmented by IL-2 stimulation. Mononuclear cells from the pleural cavity of two of these patients also had spontaneous LAK activities. Also, MNCs from the pleural cavity could be augmented by IL-2 stimulation. This finding suggests that lymphocytes circulating in some patients with lung cancer might already have activated their LAK activities. We reported previously that MNCs from the peripheral blood of some lung cancer patients showed spontaneous LAK activities without IL-2 stimulation.\textsuperscript{28} When activated with IL-2, LAK activities of MNCs from patients with lung cancer were augmented. However, the LAK activity that developed in the pleural cavity lymphocytes was significantly lower than that which developed in the peripheral lymphocytes. The high potential ability of CD16\(^+\) cells to develop LAK activity has been reported.\textsuperscript{29,30} In the present study, the proportion of CD16\(^+\) cells in the pleural cavity without malignant effusion was found to be significantly less than that in the peripheral blood. The reduction of LAK activity in lymphocytes of the pleural cavity, therefore, may be caused by the differences of the population of CD16\(^+\) cells.

Lymphocytes in the pleural cavity without malignant effusion were less activated than those of peripheral blood as measured by LAK activity. However, taking note that there were proportionately more HLA-DR\(^+\) cells in the pleural cavity than in the peripheral blood, it seems possible that they might have been activated as defined by presence of some other types of activity. Ishii et al\textsuperscript{41} reported that HLA-DR\(^+\) cells could be activated to produce tumor necrosis factor in the pleural effusions of patients with lung cancer. This finding suggests that HLA-DR\(^+\) cells may have immunologic functions in the pleural cavity preceding invasion by malignant cells. It will, therefore, be of interest to examine the immunologic functions of HLA-DR\(^+\) cells (for example, the production of tumor necrosis factor) other than LAK activity.

Human LAK development has been found to be upregulated or downregulated by autologous monocyte/macrophages.\textsuperscript{19-22,42-44} We found previously that in healthy donors, intact blood monocytes could upregulate LAK development, whereas monocytes incubated with endotoxin inhibited the development of human LAK activity.\textsuperscript{20} The macrophages in malignant pleural effusion and ascitic fluid of patients with ovarian cancer and the blood monocytes of patients with melanoma suppress LAK development.\textsuperscript{45,46} We have found that fresh human alveolar macrophages suppress LAK development.\textsuperscript{41} These findings suggest that monocyte/macrophages play an important role in LAK development. In the present study, both macrophages obtained from the pleural cavity of lung cancer patients and intact peripheral blood monocytes augmented the development of IL-2-developed LAK activity of autologous lymphocytes in the pleural cavity and the peripheral blood. Macrophages in the lung...
and the pleural cavity seem to have matured and/or are differentiated from bone marrow precursors through monocytes in the blood. The present findings suggest that macrophages in the pleural cavity of patients with lung cancer may behave like intact peripheral blood monocytes and unlike alveolar macrophages and peripheral blood monocytes of patients with melanoma. The difference in immunologic behaviors of pleural cavity macrophages and peripheral blood monocytes between alveolar macrophages may be influenced by the environment. Pleural macrophages and peripheral blood monocytes were separated from enclosed space, but alveolar macrophages were separated from the airway where they were always exposed to open air. There was no relationship in individual patients between the augmentation of LAK activity by macrophages and the phenotypes of pleural cavity lymphocytes expressing CD3, CD4, CD8, CD16, or HLA-DR (data not shown).

Lymphocytes in the pleural cavity without malignant pleural effusion could be developed by LAK activity and this activity was augmented by pleural cavity macrophages. The LAK activity developed by the pleural cavity lymphocytes was significantly lower than that developed by the peripheral blood lymphocytes. However, they can change their populations to include cells with higher activities on exposure to IL-2 against the invasion of lung cancer cells into the pleural cavity. Thus, the population of lymphocytes in the pleural cavity of patients with lung cancer without malignant effusion was different from those in malignant pleural effusion.

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