Defects in Natural Killer Cell Activity and Interferon Response in Human Lung Carcinoma and Malignant Melanoma


Departments of Medicine [W. L. S., A. D. B., A. J. J., and J. H. S.] and Surgery [R. C. D.], University of New Mexico School of Medicine, and the Albuquerque Veterans Administration Hospital [J. H. S.], Albuquerque, New Mexico 87131

ABSTRACT

The natural killer (NK) activity of peripheral blood mononuclear cells from 25 patients with squamous cell carcinoma of the lung, malignant melanoma, or epithelioid cancers of the gastrointestinal tract was measured by the lysis of 51Cr-labeled K562 target cells. NK activities of many patients with lung cancer or malignant melanoma were decreased relative to normal controls. This abnormality was significantly correlated with advancing stage of disease and the percentage of monocytes in the cell suspensions. Addition of indomethacin or removal of monocytes did not restore depressed NK function to normal levels. Abnormalities of NK function did not appear to be secondary to the presence of mononuclear suppressor cells. The response to interferon was also impaired in patients with advanced disease. The number of effector:target conjugates was normal even in patients with depressed NK function; however, the number of active lytic effectors was decreased. These results imply that the cells which bind tumor targets are present in patients with advanced cancers, but these cells are either immature or functionally inactive.

INTRODUCTION

Natural cytotoxicity is now recognized as a potentially important mechanism of immunological surveillance (19). NK cells can lyse certain tumor targets to which they have never been exposed, as well as a host of virus-infected cells (5, 8, 16). NK cells respond to IFN with increased lytic activity (32) and actually produce IFN themselves (29). The NK cell belongs to a subset of large granular lymphocytes (28, 29) and has been characterized in part with monoclonal antibodies (1, 35). It possesses Fc receptors (12) and can be modulated by a number of soluble factors (22, 23).

NK activity has been shown to be abnormal in various human diseases, including systemic lupus erythematosus (10) and cancer (13, 21). In human cancers, the importance of NK activity is unclear. However, in animals, NK activity appears crucial in the rejection of some tumors (26) and in prevention of tumor metastasis (7). Patients with advanced cancers tend to have decreased NK function (9, 21), but the etiology of this defect is uncertain. It is not known whether suppressor cells are inhibiting NK cell activity or if other mechanisms are involved.

The results reported here demonstrate that NK activity is decreased in patients with lung cancer and malignant melanoma. These abnormalities are clearly related to the clinical stage of the disease. Response to IFN is normal, except in some patients with advanced disease. The numbers of cells forming effector-tumor conjugates are normal, implying that the decreased NK activity in cancer patients is related to maturation defects or inactivation of the NK effector subpopulation.

MATERIALS AND METHODS

Subjects. Twenty-five cancer patients and 24 normal controls were studied. All patients included in this study were followed at the University of New Mexico and Albuquerque Veterans Administration Medical Center Oncology and Surgery Clinics. Twelve patients with squamous carcinoma of the lung, 11 with malignant melanoma, and 2 patients with epithelioid carcinomas of the gastrointestinal tract were studied. No patients were receiving active therapy. Because patients with different tumor types were being compared, a score of tumor stage was made: 0, no evidence of active tumor; 1, small, localized tumor; 2, locally invasive or local metastasis; and 3, massive tumor or distant metastasis.

PBMC. PBMC were isolated from heparinized whole blood by centrifugation on Ficoll-Hypaque gradients (3). Cells from the interface were washed 3 times with phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.012% KH2 PO4, and 0.16% Na2HPO4, pH 7.4) and resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) with penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% heat-inactivated human AB serum. Cell viability was greater than 99% as determined by trypan blue exclusion and contained from 2 to 40% monocytes by benzidene peroxidase staining (34). PBMC at 5 x 106 cells/ml were resuspended in RPMI 1640 medium containing 10% human AB serum, placed in glass wool columns (0.5-g glass wool in a 10-ml plastic syringe barrel), and incubated for 30 min at 37°. The glass wool-nonadherent cells were eluted with 15 ml of warm medium. The final suspension contained 0 to 1% monocytes as determined by peroxidase staining. In some experiments, PBMC from cancer patients were added to normal control PBMC (2.5 x 106 cells/well) in microtiter plates at the following cancer:control cell ratios: 0:1; 0.2:1; 0.4:1; 0.6:1; and 1:1. These suspensions were then cultured for 16 hr prior to placement in the NK assay.

Drug Treatment of PBMC. Indomethacin (Sigma) was dissolved in 95% ethanol at 10 mg/ml. The solution was then diluted with RPMI 1640 as necessary to achieve the appropriate final concentrations. The small amount of ethanol in the cultures did not affect cell viability or NK function. Indomethacin at 1 µg/ml was incubated with PBMC for 16 hr at 37°. The cells were washed with media prior to use in subsequent assays. PBMC were incubated with n-interferon (NIH standard, G023-90X-577; Bethesda, MD) at 0 to 1000 units/ml at 37° for 30 min. Optimal stimulation of NK activity as measured in LU was achieved with 500 units of IFN/ml. Percentage of enhancement of NK activity was defined as:

\[
\frac{(\text{LU}_{\text{nu}}/10^7 \text{ cells} - \text{LU}_{\text{base}}/10^7 \text{ cells}) \times 100}{\text{LU}_{\text{base}}/10^7 \text{ cells}}
\]
NK Activity in Human Cancer

**Supernatants.** Normal control and cancer PBMC in RPMI 1640 with 10% AB serum were cultured at 1.25 x 10^6 cells/ml for 16 hr. The supernatant was collected and then incubated with fresh effector cells from normal controls at 1.25 x 10^6 cells/ml of supernatant for 2 hr prior to placement in the NK assay.

**NK Assay.** K562 (17) is a standard target for the human NK assays and was used in this study. Because NK activity may vary somewhat from day to day, a normal control sample was run with each patient sample. The medium used for continuous culture was RPMI 1640, supplemented with 10% fetal calf serum. Target cells at 5 x 10^6/0.2 ml of fetal calf serum were incubated with 0.1 ml Na^5^CrO_4_ (specific activity, 1.0 mCi/ml; New England Nuclear, Boston, MA) for 45 min at 37°. The cells were washed 3 times with RPMI 1640 containing AB serum and then placed into wells of round-bottomed microtiter plates (Linbro, Ham- stead, CT). Various numbers of effector cells were added to 10 x 10^6 labeled target cells, such that effector:target ratios were 100:1, 50:1, and 25:1. The final volume in each well was 0.2 ml. The plates were incubated for 4 hr at 37° and then centrifuged at 100 x g for 10 min. Supernatant (0.1 ml) was removed and placed in glass tubes and counted in an automatic y-scintillation counter. Spontaneous release was determined with a series of wells containing no effector cells. Maximum release was obtained by treating a series of wells with Lyzerglobin (J. T. Baker Chemical Co., Bethlehem, PA). NK activity was calculated as:

\[
\% \text{ of lysis} = \frac{\text{Experimental } ^5\text{Cr release} - \text{spontaneous } ^5\text{Cr release}}{\text{Maximum } ^5\text{Cr release} - \text{spontaneous } ^5\text{Cr release}} \times 100\%
\]

A LU was defined as the number of effector cells necessary to result in a 30% lysis of 1.0 x 10^6 labeled targets. The relative NK activity was expressed as LU/10^7 cells. These values were determined from individual effector:target titration curves. Effector:target conjugates were quantitated in a manner similar to that described by Targan et al. (27) using an agarose single-cell assay with glass wool-nonadherent PBMC.

**Statistical Methods.** The 2-tailed Student's t test was used to determine the significance of differences between the means of 2 groups. Linear regression analysis was used to compute linear correlation coefficients (r values) and p values between 2 variables.

**RESULTS**

**NK Function in Human Cancer.** NK activity (expressed as percentage of lysis) in cancer patients as a group was significantly decreased relative to normal controls (Chart 1) at 3 different effector:target ratios (i.e., at a 50:1 ratio: controls, 49.8 ± 13.5% (S.D.); cancer patients, 38.0 ± 22.5%). All 24 normal controls demonstrated NK activity greater than 17 LU/10^7 cells. Of the 25 cancer patients studied, 12 had significantly decreased NK function (Table 1). Depressed NK activity was defined as less than 17 LU/10^7 cells. Conversions to LU/10^7 are seen in Table 2. The NK activity of the lung carcinoma (n = 12) and malignant melanoma (n = 11) groups was similar: lung carcinoma, 48.1 ± 10.1 LU/10^7 cells; melanoma, 44.3 ± 9.2 LU/10^7 cells (p > 0.10).

In addition, the clinical stage correlated with decreases in NK activity (r = 0.56; p < 0.01), as can be seen from Chart 2. A similar correlation with progressive stage existed with melanoma (r = -0.63; p < 0.05) and lung carcinoma (r = -0.90; p < 0.01). Interestingly, patients with limited tumor (Stage 0 to 1) appeared to have normal or actually accentuated NK activity. Those with extensive tumor had markedly impaired NK activity (p < 0.05). Normal controls varied in NK activity when measured on subsequent days by 11.5 ± 6.4 LU/10^7 cells, while cancer patients varied by 9.8 ± 7.33 LU/10^7 cells (p > 0.05). Thus, there was excellent reproducibility with variations in individual NK activity of less than 20%. Patients with impaired NK activity continued to have impaired NK activity when measured on subsequent days. Overnight incubation of effector cells did not increase the
NK activity of cancer PBMC: cancer, 40.0 ± 12.3 LU/10^7 cells; control, 61.3 ± 11.8 LU/10^7 cells (p < 0.05). The percentage of monocytes in PBMC was increased in cancer patients relative to normal controls: cancer, 16.2 ± 12%; control, 11.8 ± 7.0%), but removal of glass wool-adherent cells from suppressed cancer patients (n = 12) did not restore depressed NK activity to normal levels. The NK activity of glass wool-nonadherent PBMC from NK-depressed cancer patients was 28.3 ± 15.7 LU/10^7 cells, while that of glass wool-nonadherent cells from controls was 54.2 ± 16.2 LU/10^7 cells (p < 0.05). The percentage of monocytes found in PBMC from cancer patients correlated with both the stage of disease (r = 0.507; p < 0.05) and a decrease in observed NK activity (r = -0.66; p < 0.05).

Prostaglandins have been implicated in monocyte-mediated suppression of lymphocyte function (6). Indomethacin, a potent prostaglandin synthetase inhibitor, was added to cultures as described in "Materials and Methods." Although indomethacin treatment resulted in an increase in NK activity of controls, it did not restore decreased NK function in cancer patients to normal: cancer patients with depressed NK function after indomethacin treatment, 28.0 ± 12.6 LU/10^7 cells; controls after indomethacin, 60.0 ± 18.1 LU/10^7 cells (p < 0.01). Normal PBMC treated with supernatants from 24-hr cultures of PBMC from controls and cancer patients resulted in no inhibition of NK activity: control, 16.9 ± 7.1; cancer, 30.6 ± 15.0 (p < 0.05). Patients with normal NK activity possessed a similar number of active conjugates (10.8 ± 5%) as compared to controls (5.7 ± 0.7%) compared to normal controls (12.6 ± 5.6% (p < 0.05). Patients with normal NK activity possessed a similar number of active conjugates (10.8 ± 5%) as compared to normal controls (5.7 ± 0.7%) compared to normal controls (12.6 ± 5.6% (p < 0.05).

**Single-Cell Assay.** The percentage of effector-target conjugates was determined in 8 patients with impaired NK activity and in 8 normal controls (Table 6). As can be seen, NK activity was markedly impaired in these patients relative to controls (p < 0.05); however, conjugate formation was the same for both groups. Interestingly, the percentage of active lytic effectors in conjugates of patients with impaired NK activity was decreased (5.7 ± 0.7%) compared to normal controls (12.6 ± 5.6% (p < 0.05). Patients with normal NK activity possessed a similar number of active conjugates (10.8 ± 5%) as compared to controls (5.7 ± 0.7%) compared to normal controls (12.6 ± 5.6% (p < 0.05).

**Effect of Interferon.** Cancer patients and controls responded to IFN as shown in Table 3. As can be seen, relative enhancement was 75.1 ± 14.0% in cancer patients, compared to 180.0 ± 27% in normal controls (p < 0.05). Thus, cancer patients did not respond to IFN as well as did normal controls. Of 25 cancer patients studied, 12 had an impaired response to IFN. As a group, there was no correlation between clinical stage and the response to IFN. However, it was evident that the cancer patients could be separated into 2 groups: those with limited tumor and those with extensive tumor (Table 3). Although similar relative enhancement by IFN was present in both groups, base-line NK activity was markedly enhanced in those with limited tumor and suppressed in those with extensive tumor. Those with limited tumor responded to IFN in such a manner that there was no difference in NK activity compared with normal controls (Table 3). Patients with large tumor burdens did not achieve normal levels of NK activity with IFN exposure. Table 4 shows that variable incubation times with IFN did not significantly alter the impaired response to IFN. Similarly, increasing concentrations of IFN did not overcome the defect in IFN-induced NK enhancement (Table 5). Thus, response to IFN appeared to be impaired in cancer patients with extensive disease. Cancer patients with a limited tumor burden, however, achieved normal levels of NK activity after IFN exposure. In addition, some patients with a limited tumor burden actually had an accentuation of base-line NK activity.

**Table 3**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Base-line NK activity (LU/10^7 cells)</th>
<th>IFN-induced NK activity (LU/10^7 cells)</th>
<th>% of enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cancer (n = 25)</td>
<td>50.9 ± 11.4^a</td>
<td>90.4 ± 20.0</td>
<td>75.1 ± 21</td>
</tr>
<tr>
<td>Cancer with limited tumor (n = 9)</td>
<td>111.5 ± 16.7</td>
<td>162.6 ± 46.6</td>
<td>45.8 ± 35</td>
</tr>
<tr>
<td>Cancer with extensive tumor (n = 16)</td>
<td>16.9 ± 7.1</td>
<td>30.6 ± 15.0</td>
<td>93.0 ± 25</td>
</tr>
<tr>
<td>Normal controls (n = 24)</td>
<td>57.1 ± 10.5</td>
<td>150.0 ± 25.0</td>
<td>180.0 ± 27</td>
</tr>
</tbody>
</table>

^a IFN concentration was 200 units/ml.

% of enhancement = (LUinFNa-induced − LUbase-line)/LUbase-line × 100

^-b Mean ± S.D.

**Table 4**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Base line (LU/10^7 cells)</th>
<th>IFN (LU/10^7 cells)</th>
<th>% of enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>57.0 ± 15.6^a</td>
<td>61.0 ± 25.0</td>
<td>31.2 ± 23.0</td>
</tr>
<tr>
<td>4 hr</td>
<td>55.0 ± 24.2</td>
<td>80.6 ± 31.7</td>
<td>55.0 ± 21.0</td>
</tr>
<tr>
<td>16 hr</td>
<td>55.3 ± 23.8</td>
<td>83.6 ± 31.2</td>
<td>59.0 ± 35.0</td>
</tr>
<tr>
<td>Normal control (n = 24)</td>
<td>57.1 ± 10.5</td>
<td>150.0 ± 25.3</td>
<td>180.0 ± 27.0</td>
</tr>
</tbody>
</table>

^a Mean ± S.D.
Cancer patients 57.6 ± 26.8* 65.2 ± 21.0 71.7 ± 36.3 79.8 ± 34.3

Total number of active lytic effectors, although the total number activity in human cancer appears to be related to a decreased function seemed to decline with advancing disease. The implication of these findings is unknown. Manna and Burton (7) have found that NK effectors may explain some of the decreased NK activity of patients advanced disease (4). Thus, immune complex inactivation of NK function then results in further metastatic spread is not known.

In systemic lupus erythematosus, a similar defect in NK activity has been described (10). This also appears to be related to disease activity and, in addition, to the presence of circulating immune complexes (14). The NK cell has been shown to possess Fc-IgG receptors, and triggering of these receptors can result in a decrease in NK activity (20). Immune complexes have been found in patients with cancer, and particularly in those with advanced disease (4). Thus, immune complex inactivation of NK cells may explain some of the decreased NK activity of patients with progressive cancer. Merrill et al. (20) have reported that 24-hr incubation will remove immune complex-mediated inhibition of NK activity. However, in our study, 24-hr incubation did not restore NK function to normal in suppressed cancer patients. This implies that more complicated abnormalities than simple immune complex inhibition are present in cancer patients.

Our study demonstrated that the presence of an increased number of monocytes in the PBMC suspension was associated with a decreased NK function and progressive disease. Enrichment of monocytes in PBMC from cancer patients has been described previously (11). Monocytes have been shown to suppress lymphocyte function and to secrete prostaglandins (6) which are potent inhibitors of NK function (2). In our study, however, removal of glass wool-adherent cells and addition of indomethacin did not restore depressed NK function in cancer patients. The increase in monocytes was probably another measure of disease progression and was not a direct cause of the abnormal NK function observed in cancer patients. Furthermore, no evidence of suppressor cell inhibition of NK function was found through coculture experiments. This was supported by the lack of inhibition of NK activity by addition of supernatants from cultured PBMC obtained from cancer patients.

The observed defect in NK activity of cancer patients was not associated with a decrease in effector:target conjugate formation. Approximately 20 to 50% of tumor-binding lymphocytes have the capability of lysing the K562 target in normal individuals (27, 32). The finding of normal effector:target conjugate numbers in cancer patients implies that potential NK cells are not adequately differentiated to affect lysis or that they are inactivated in some manner. The exact mechanisms of these abnormalities are uncertain.

The response to IFN was decreased in many patients with extensive tumor. Patients with limited tumor burdens achieved normal levels of NK activity with IFN exposure, while those with extensive tumor did not (Table 3). IFN acts to augment NK activity by recruiting pre-NK cells and by increasing recycling of existing NK cells (32). To determine which mechanism was predominant, an analytical assay, such as described by Ullberg et al. (31), was necessary. The single-cell assay demonstrated that effector:tumor conjugate formation was normal; however, those patients with advanced disease did not have as many active lytic target binding cells. This finding was very similar to that of Katz et al. (15) in systemic lupus erythematosus. Recently, Sibbitt et al. (24) have reported that the impairment of NK activity in systemic lupus erythematosus appears to be related to impaired release of a soluble cytotoxic factor, thus implying a specific cellular defect. A similar mechanism may be present in cancer patients. Kaddish et al. (13) have demonstrated that IFN production is normal in patients with advanced carcinomas. Thus, base-line NK function and IFN response are impaired in patients with progressive disease without an abnormality in IFN production. This finding is in contrast to the work of Lucero et al. (18), who reported a normal response to IFN in tumor-bearing patients. In that study, the authors did not subgroup the patients into stages; thus, the effect of progressive disease was not described. Interestingly, in the present study, cancer patients with a limited tumor burden actually had an increase in NK activity, relative to normal controls (Table 3). This is an interesting phenomenon and may represent a host response to the tumor. Indeed, IFN can be generated by effector:target contact and could result in the increase in NK activity noted in this group (30).

<table>
<thead>
<tr>
<th>Patients</th>
<th>NK activity (LU/10^7 cells)</th>
<th>% of effector-tumor conjugates</th>
<th>% of active lytic effector cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (n = 8)</td>
<td>59.0 ± 14.0*</td>
<td>15.2 ± 5.0</td>
<td>12.6 ± 5.6*</td>
</tr>
<tr>
<td>Cancer (n = 8)</td>
<td>15.1 ± 3.3</td>
<td>16.1 ± 7.4</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>p</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

a Mean ± S.D.

b Mean ± S.D.
Our study demonstrates that NK activity is often decreased in malignant melanoma and lung cancer patients. This defect is related to the extent of disease progression, but is not associated with cell-mediated suppressive mechanisms. In general, response to IFN is normal in cancer patients, except those with advanced disease. There does not seem to be a depletion of tumor-adherent lymphocytes in cancer patients with decreased NK activity; rather, there is a decrease in active lytic tumor-binding effector cells. These findings imply a defect in maturation of cells capable of natural cytotoxicity or inactivation of existing NK cells. Whether circulating tumor antigens or antigen-antibody complexes (33) are responsible for this abnormality is unknown at this time.

REFERENCES

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