Decreased Natural Killer Cell Activity in Children with Untreated Acute Leukemia

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ABSTRACT

Natural killer cell activity was evaluated in children with acute lymphocytic and acute myelogenous leukemia. Peripheral blood mononuclear cells isolated at the time of diagnosis and before initiation of therapy were mixed with 51Cr-labeled K562 or MOLT-4 target cells at a ratio of 100:1. In 13 consecutive cases of acute lymphocytic leukemia, the mean percentage of lysis of K562 cells (15.0%) was significantly below that of adult (49.8%) and age-related controls (35.9%). A similar pattern was observed against MOLT-4 targets (acute lymphocytic leukemia, 11.3%; adults, 39.8%; and pediatric controls, 28.4%). The mean activity in 8 cases of acute myelogenous leukemia was also markedly reduced (6.8% versus K562 and 6.0% versus MOLT-4). Linear regression analyses of white blood cell, lymphocyte, and leukemia blast counts failed to demonstrate any correlation between peripheral cell counts and natural killer cell activity. Thus, it would not appear that the observed decrease in lysis was due merely to dilution of effectors with blasts. The lytic activity of cells isolated from patient blood was significantly lower than that from cells isolated from an equal volume of blood from a normal adult. These results suggest that the decreased natural killer cell activity is not explained by simple dilution. Instead, they indicate an absolute decrease in lytic potential. Additional experiments have precluded suppressor cell involvement and competitive inhibition of blasts with target cells as possible causes for depressed lysis.

INTRODUCTION

The existence of a cell population which, without prior sensitization, can selectively lyse certain tumor cells in vitro is now well established. This heterogeneous population, most often called NK cells,3 is found in a variety of animals including mice, rats, chickens, guinea pigs, hamsters, and humans (4, 15, 17, 18, 23, 25, 33, 35). In humans, the majority of these effector cells are lymphocytes, probably of the pre-T-cell lineage (34).

Several investigators have demonstrated a correlation between levels of natural cytotoxicity and tumor resistance in vivo (13, 14, 24, 27). For example, Hanna and Fidler (13, 14) have shown using various murine tumor models that in many instances NK cell activity is correlated with resistance to tumor metastatic potential. In addition, Ojo (24) has demonstrated a positive correlation between NK cell levels and in vivo resistance to tumor growth. In humans with Chediak-Higashi syndrome (a disease with an increased incidence of hematological cancers), NK cell function is severely depressed (12).

The possible involvement of NK cells in immune surveillance has led to the study of this effector mechanism in various disease states including multiple sclerosis and immunodeficiency (5, 30). Others have shown the effect of cancer on NK cell levels (11, 16, 29). We evaluated NK cell activity in children with ALL and AML and found a marked decrease in cytotoxicity in these patients. Possible reasons for the differences found in these groups were also examined.

MATERIALS AND METHODS

Patients. Thirteen consecutive patients with ALL (mean age, 4.3 years; range, 1 to 9 years) and 8 consecutive patients with AML (mean age, 6.2 years; range 1 to 15 years) were the subjects of the present study. Diagnoses were based on standard morphological and cytochemical criteria as well as various immunodiagnostic markers (terminal deoxynucleotidyl transferase, common ALL antigen, erythrocyte rosettes, and surface immunoglobulin). Morphological classification was standardized according to the French-American-British criteria applied to the initial diagnostic bone marrow aspirates (6). The pediatric control group was composed of 9 children (mean age, 7.8 years; range, 5 to 15 years) who were admitted to the hospital for elective surgery and who were free of any known viral or bacterial infections.

Effector Cell Separation. At the time of diagnosis and prior to the initiation of therapy, 10 ml of peripheral blood were obtained by venipuncture in sterile heparinized vacutainers. An adult control was drawn at the same time. Since 2 samples were obtained via overnight air delivery and in order to handle all specimens in a consistent manner, all blood samples (patient and controls) were tested 8 to 12 hr after collection. However, initial studies demonstrated no difference in NK cell activity when tested immediately or 24 hr after collection.

The PBM cells were isolated using a modification of a technique originally reported by Böyum (9). Briefly, the peripheral blood was diluted 1:1 with sterile PBS and then overlaid on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, N. J.). Following centrifugation at 18° for 40 min, the mononuclear cells were carefully collected from the Ficoll-serum interface, washed 2 times in PBS, and resuspended in 1 ml of C-RPMI. The cell suspension was diluted to 5 x 106 cells/ml with C-RPMI.

Target Cells. The K562 cell line was originally isolated from a patient with chronic myelogenous leukemia (20) but is now thought to be an erythroleukemic cell line (3). The MOLT-4 cell line was isolated from a human T-cell lymphoma (22). Each target cell line was maintained in C-RPMI and was split 24 to 48 hr prior to use in an NK cell assay. The target cells were labeled by incubating approximately 106 cells in 0.5 ml of medium with 100 μCi of 111In (sodium chromate) for 40 min at 37° with occasional shaking. The labeled cells were washed 3 times in warm medium, counted, and adjusted to a concentration of 2 x 106 cells/ml.

NK Cell Assay. The NK cell activity was measured by the 51Cr release

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3 The abbreviations used are: NK cell, natural killer cell; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; PBM cells, peripheral blood mononuclear cells; PBS, Dulbecco’s phosphate-buffered saline without calcium and magnesium (KH2PO4, 0.2 g/liter), KCl (0.2 g/liter), NaHPO4 (1.15 g/liter), and NaCl (8.0 g/liter), pH 7.2 to 7.4; C-RPMI, Roswell Park Memorial Institute Tissue Culture Medium 1640 with 10% heat-inactivated fetal bovine serum, 20 mm 4-(2-hydroxyethyl)-1-piperazinenuclear acid buffer, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 2 μM glutamine; E:T ratio, effector cell to target cell ratio.
4 Received March 22, 1983; accepted August 8, 1983.

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assay described by Kiessling et al. (17). Each effector cell suspension was plated in triplicate. Briefly, 200 µl of the appropriately diluted cell suspension (usually 5 x 10⁶ cells/ml) were mixed with 50 µl of ⁵¹Cr-labeled target cells, yielding an E:T ratio of 100:1. After a 4-hr incubation at 37° in a humidified atmosphere containing 5% CO₂, the cells were pelleted by centrifugation for 10 min at 250 x g. One hundred µl of supernatant were removed from each well, and the amount of radioactive isotope present was determined using a γ scintillation counter. The percentage of lysis was determined using the formula:

\[
\% \text{ of lysis} = \frac{cpm \text{ sample} - cpm \text{ AC}}{cpm \text{ MR} - cpm \text{ AC}} \times 100
\]

where AC (autologous control) was a mixture of nonlabeled plus labeled target cells at a 100:1 ratio and MR (maximum release) was determined by incubating labeled target cells in 1% Triton X for 4 hr.

HNK-1 Monoclonal Antibody. Leu-7 (Becton Dickinson and Co., Sunnyvale, Calif.) was used to detect NK cells. This monoclonal antibody, referred to as HNK-1 originally (1), has been reported to recognize the large granular lymphocyte population in which the NK cells reside (2).

Immunofluorescence Assay. Leu-7-reactive cells were enumerated using indirect membrane immunofluorescence. Briefly, 5 x 10⁶ cryopreserved patient or control PBM cells were incubated at 4° with 2.5 µg of Leu-7 in a volume of 0.1 ml of C-RPMI. After 30 min, the cells were washed 3 times in PBS containing 0.1% sodium azide. They were then incubated at 4° with 100 µl fluorescein-conjugated goat anti-mouse IgM (Tago, Inc., Burlingame, Calif.).

After 30 min, the cells were again washed 3 times, and a wet mount was examined using a Nikon Labophot microscope with an epifluorescent-attachment. Two hundred cells per slide were counted, and the percentage of Leu-7-positive cells was recorded.

Activity per Unit Volume of Blood. In addition to comparing lytic activity per 10⁶ cells, the activity present in cells isolated from equal volumes of patient and adult control peripheral blood was also determined. Ten ml of blood were obtained from a normal adult as well as a newly diagnosed patient having a WBC of 58,000 cells/cu mm. The PBM cells from each sample were isolated as usual, and the nucleated cell concentrations were determined. The control suspension was diluted from its original concentration (10.5 x 10⁹) to 5 x 10⁹ cells/ml, a dilution factor of 2:1. The patient’s PBM cell suspensions were diluted by the same factor. This resulted in a cell concentration much higher than that of the control (approximately 10 times that of the control). In order to control for the difference in cell concentrations, viable cryopreserved lymphoblasts were added to the control suspension such that the total cell concentrations in each sample were equal. These were then plated at 1:1, 1:2, and 1:4 dilutions. The samples were also plated normally (at E:T ratios of 100, 50, and 25:1). After a 16-hr incubation period, the percentage of lysis was determined as described earlier.

Cold Target Inhibition. Viable cryopreserved leukemic cells, previously isolated from newly diagnosed patients, were washed and diluted to 5.0, 2.5, 1.25, and 0.6 x 10⁶ cells/ml. One hundred µl of each of these were mixed with 100 µl of effector cells at 5.0 x 10⁶ cells/ml. Each admixture was plated in triplicate with 50 µl of ⁵¹Cr-labeled K562 target cells. This resulted in a constant E:T ratio of 50:1 and effector/inhibitor ratios of 1:1, 1:2, 1:4, and 1:8. In addition, unlabeled K562 cells which had been maintained in culture or which had been cryopreserved were used as positive controls for competitive inhibition of lysis. The resulting lysis from each group of cell mixtures was compared to that of normal effectors plated as usual at an E:T ratio of 50:1. Results were reported as percentage of inhibition, which was calculated by the formula:

\[
\% \text{ of inhibition} = 1 - \frac{\% \text{ of lysis of effector mixture}}{\% \text{ of lysis of effectors alone}} \times 100
\]

Statistical Analysis. The significance of differences between the means of the various groups was evaluated by parametric and nonparametric methods (Student’s t test and the Mann-Whitney test (36), respectively). Linear regression analysis was used for the statistical analysis of the correlation between circulating cell counts and percentage of lysis.

RESULTS

The natural cytotoxicity of cells obtained from the blood of newly diagnosed patients was measured against K562 and MOLT-4 target cells (Table 1). Using Student’s t test, the mean level of NK cell activity versus K562 targets from the 13 children with ALL [15 ± 12.4 (S.D.)] was shown to be significantly lower (p < 0.01) than either the adult (49.8 ± 11.2) or pediatric (35.9 ± 11.3) control groups. Nonparametric analyses of these data as well as of the data in Table 2 resulted in even greater levels of significance (p < 0.005). As reported previously (7, 10), the level of activity between normal adults and children did not differ significantly. It is interesting to note that while the patient group as a whole exhibited low cytotoxicity, there were some patients which demonstrated at least low normal activity (Patients 5, 9, 11, and 12). It may be important to compare these patients to those with low NK cell activity to determine if the higher level of NK cell activity correlates with less morbidity and/or prolonged survival.

The NK cell activity versus the MOLT-4 cell line yielded results similar to those obtained using K562 target cells. The patient group had 11.3 ± 8.9% lysis whereas the means of the adult and pediatric controls were 3.98 ± 16.1% and 28.4 ± 12.7%, respectively (p < 0.01). Furthermore, the same 4 patients discussed above exhibited the highest NK cell levels of the group. While the results obtained with K562 and MOLT-4 targets do show some minor variation, we feel the variation is most probably a reflection of the day-to-day variation of the assay (perhaps due to daily fluctuation in target cell sensitivity) rather than an indication of effector cell heterogeneity. The report that the target cell binding site on both the K562 and the MOLT-4 cell line are the same supports such an explanation (31).

The mean percentage of lysis of K562 cells from the AML group was even more depressed than that of the ALL group (Table 2). The activity from these patients was 6.8 ± 7.1% as compared to adult controls, 43.6 ± 11.1%. The difference between the patient and either adult or pediatric controls was statistically significant (p < 0.01). Similar results were observed when the MOLT-4 cells were used as targets.

Although the difference in cytotoxicity was clearly established, it was not apparent whether this difference was absolute, i.e., due to a decrease in function per unit volume of blood, or relative, i.e., due to a decrease in activity per 10⁶ nucleated cells. We tried to ascertain if the decrease in activity could be associated with the abnormally high concentration of nucleated cells (often associated with acute leukemias) which subsequently would decrease the ratio of NK cells to non-NK cells in the PBM cell suspension. We approached this problem in 2 ways. The concentration of nucleated cells in the peripheral blood at the time of diagnosis was compared with the level of cytotoxicity. Although we found no correlation in the normal population between either the WBC or the lymphocyte count and NK activity (correlation coefficients of -0.43 and +0.08, respectively), one still could argue that such a correlation could still exist in children with leukemia. If the loss of activity were merely due to dilution, one would expect to find an inverse correlation between WBC, lymphocyte count, and/or lymphoblast count and NK cell activity. As exemplified in Charts 1 and 2, this was not the case. Linear
Table 1
Natural cytotoxicity of ALL patients at diagnosis

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<th>FAB classification</th>
<th>Patient</th>
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<th>Pediatric control</th>
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<th>Adult control</th>
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<td>15.0 ± 12.5%</td>
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<td></td>
<td>11.3 ± 8.9%</td>
<td>39.8 ± 16.1%</td>
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<td>35.9 ± 11.3%</td>
<td>28.4 ± 12.7%</td>
<td></td>
<td>15.0 ± 12.5%</td>
<td>49.8 ± 11.2%</td>
</tr>
</tbody>
</table>

* FAB, French-American-British classification of the predominating blast type at diagnosis; NT, not tested.
* Patient identification number.
* PBMC cells were mixed with 51Cr-labeled target cells at a ratio of 100:1 and incubated for 4 hr at 37°C.
* Adult controls and patients were collected and were run simultaneously.
* Pediatric controls were collected and were run as they became available.
* Mean ± S.D.

Table 2
Natural cytotoxicity of AML patients at diagnosis

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<th>Pediatric control</th>
<th>Patient</th>
<th>Adult control</th>
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<tr>
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<td>6.8 ± 7.1%</td>
<td>43.6 ± 11.1%</td>
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<td>6.0 ± 7.4%</td>
<td>36.4 ± 5.4%</td>
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<td>35.9 ± 11.4%</td>
<td>28.4 ± 12.7%</td>
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<td>6.0 ± 7.4%</td>
<td>36.4 ± 5.4%</td>
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</table>

* FAB, French-American-British classification of the predominating blast type at diagnosis.
* Patient identification number.
* PBMC cells were mixed with 51Cr-labeled target cells at a ratio of 100:1 and incubated for 4 hr at 37°C.
* Adult controls and patients were collected and were run simultaneously.
* Pediatric controls were collected and were run as they became available.
* Mean ± S.D.
regression failed to demonstrate any correlation of NK cell activity with any of the 3 cell counts. The correlation coefficients for the WBC, lymphocyte, and blast counts were −0.23, −0.29, and −0.04, respectively.

Additionally, the number of lytic units per ml of blood from some patients with counts greater than 30,000 was found to be lower than the number of lytic units of controls. These data indicate loss of activity was not relative to the number of circulating leukemic cells.

Since this approach only suggests that the decreased activity was absolute, we next compared the lytic activity in a unit volume of patient blood and of a control. PBM obtained from a patient and control were diluted identically, yielding a cell concentration in the patient sample that was almost 10-fold greater than the control. "Filler" cells were then added to the control suspension to yield a concentration equal to that in a patient suspension. These preparations were then plated at full strength, or diluted 1:2 and 1:4. As seen in Table 3, the NK activity was expectedly low in both the patient (10.5%) and Patient' (7.9%) groups. The activity in the Control' (8.7%) was lower than that of the control (69.9%), but the level of activity approached normal when more media was provided for the cells (at 1:4; control, 53.5% versus Control' 37.7%); in contrast, the level of NK cell activity remained severely depressed in the patient suspension even after dilution. The results of this experiment indicate that there was some steric hindrance evident in the control lymphoblast mixture. More importantly, this may demonstrate an absolute decrease in NK cell activity in ALL.

Cold Target Inhibition. The observed reduction in natural cytotoxicity in the leukemic blood could be due to active suppression or "passive" competitive inhibition mediated by some or all of the circulating blasts. In order to examine these possibilities, effector cells from a normal control were mixed with viable, cryopreserved lymphoblasts or myeloblasts which were isolated from patients at the time of diagnosis. Fresh K562 cells were used as a positive control for inhibition.

As seen in Chart 3, there was no inhibition observed with any of the patient cells. However, one could argue that cryopreservation changed the antigenic sites recognized by the NK cells and thus removed or altered any competitive inhibition that existed prior to preservation. The addition of a second positive control was included to test for this possibility. K562 targets which had been cryopreserved 1 year earlier were also mixed with the normal effector cells. As seen in Chart 3, the inhibition by either the fresh or frozen targets was identical. Thus, it appeared that there was no competitive inhibition caused by the leukemic cells. Furthermore, the results indicate that suppressor cells are not involved.

Immunofluorescence. In order to ascertain if the decrease in cytotoxicity was attributable to a decrease in the number of effector cells, the percentage of Leu-7-positive cells in cryopreserved PBM cells from patient and adult controls was compared. In addition, the total number of Leu-7-positive cells per cu mm of blood was also calculated.

Although the percentage of NK-like cells was markedly decreased in the patient population, the total number of Leu-7-positive cells per cu mm of blood was either equal to or greater than those of the controls (Table 4).

### DISCUSSION

Several studies involving murine tumor models suggest that NK cells may be capable of mediating immune surveillance against tumors (13, 14, 24, 27). Ojo (24) has reported that mice with stimulated NK cell activity are more resistant to the growth of certain transplanted tumor cells than are unstimulated controls. Hanna and Fidler (14) have also suggested that natural cytotoxicity may inhibit the growth of metastatic tumor clones. These and similar observations have led to the study of natural cytotoxicity in various human cancers. Hersey et al. (16) have shown that families with a high incidence of melanoma have low NK cell activity. Others have shown a decrease in natural cytotoxicity associated with various human cancers (44). This decrease has been observed in advanced or metastatic cases and may be due to a defect in the rate of killing or in the recycling capacity of NK cells (37), which may in turn be attributed to the presence of suppressor cells or cytophilic antibodies (38). The present study extends these observations to children with...
themselves since the admixing of blasts and effectors failed to demonstrate the presence of active suppressor cells and/or not likely that the function is being inhibited by the leukemic cells considered, the main reason for the loss of cytotoxicity is an of the numbers of circulating leukemic cells, the data in Chart 2 from cord blood (10).”

ALL was far below that observed in infants (10) or in lymphocytes considered, the activity of all but 4 patients with either AML or ALL and AML. Although other investigators have examined these disease states (19, 21, 40), none of these reports deals with NK cell activity prior to chemotherapy. Thus, it is difficult to determine from these studies whether the observed differences are secondary to the malignant disease itself or to immunosuppression induced by therapy. By examining the level of cytotoxicity prior to treatment, we have shown that children with either ALL or AML have lower activity than their respective controls. Although the pediatric controls were not age matched, they do provide a useful point for comparison. While in some studies NK cell levels do exhibit minor fluctuations with age, these differences are not statistically significant (7, 10). Even when these fluctuations are considered, the activity of all but 4 patients with either AML or ALL was far below that observed in infants (10) or in lymphocytes from cord blood (10).* Since reduction in NK activity was shown to be independent of the numbers of circulating leukemic cells, the data in Chart 2 and Table 3 indicated that, although steric hindrance must be considered, the main reason for the loss of cytotoxicity is an absolute loss in the number of functional NK effector cells. It is not likely that the function is being inhibited by the leukemic cells themselves since the admixing of blasts and effectors failed to demonstrate the presence of active suppressor cells and/or competitive inhibition of leukemic cells with targets for the NK cell receptor. It is possible that the suppressor cells may have been selectively destroyed by cryopreservation. For example, certain macrophages have been shown to suppress NK cell activity (8, 41) and may be more cryosensitive than are lymphocytes. This possibility of selective destruction of a macrophage suppressor cell seems remote since the number of macrophages contained in the mononuclear cell suspensions isolated from children with ALL was below the 10% used to achieve suppression in the studies mentioned above (8, 41). The absolute decrease in activity could be due to one of 2 causes or their combination: a functional defect in the lytic potential of the cell (including recognition and/or lysis); or a decrease in the number of effector cells. Using a monoclonal antibody shown to react with large granular lymphocytes (the cell population in which the majority of human NK cell activity resides), we have attempted to discern which of these 2 causes apply. From Table 4, it would appear that although the percent-age of Leu-7-positive cells is lower in the patient groups, the total number of Leu-7-positive cells is no lower than in the control group. These results support the theory that a functional lytic defect is present rather than a lack of cell numbers. This defect may be a decrease in the recycling capacity of the NK cells, as has been reported in other neoplastic conditions (37). The lytic potential might also be compromised if active natural killer cytotoxic factors, as reported by Wright and Bonavida (42, 43), are lacking. However, it is possible that the Leu-7 antibody recognizes cells other than NK cells, and such an occurrence would lead to a false elevation in the number of NK cells as detected by immunofluorescence. The chances of such false positives might be diminished by the use of a more specific antibody such as Leu-11 (26) or by measuring the number of target binding cells (32).

Further study of parents and siblings of these patients may provide evidence regarding whether low NK cell activity predisposes the children to leukemia. It may also prove interesting to determine if those few patients with relatively normal NK cell activity have better prognosis than those with depressed cytotoxicity. Since NK cell activity may be reduced in adults with preleukemic states, and although results are somewhat equivocal at present, deficits in NK cell activity may precede the development of acute leukemia (28, 39).

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