ABSTRACT

The possible role that natural cell-mediated cytotoxicity may play as a host defense mechanism against malignant tumors was investigated. We measured natural cell-mediated cytotoxicity (51Chromium released) in 79 normal individuals using K562 leukemia cells as targets in quadruplicate assays after 3, 4, and 5 hr of incubation using three different effector:target cell ratios (6:2:1, 25:1, and 50:1). Natural cell-mediated cytotoxicity was significantly lower (p < 0.005) in each of the nine separate assay conditions for individuals with a high familial incidence of cancer compared to individuals with a low incidence of cancer. Moreover, natural cell-mediated cytotoxicity inversely correlated with the number of family members with cancer. The relationship between high familial cancer incidence and low natural cell-mediated cytotoxicity was observed in males as well as in females and in nonsmokers as well as in smokers. The same conclusion was reached whether the data were expressed as percentage of 51Chromium released, as lytic units per 10^6 mononuclear cells, or as lytic units per ml of peripheral blood. Thus, defects in natural cell-mediated cytotoxicity may play a role in the initial stages of human tumorigenesis. It may also be possible to identify individuals at increased risk of cancer development.

INTRODUCTION

Natural or spontaneous cytotoxicity, mediated by a subpopulation of lymphocytes, may play a central role in surveillance against tumor development. Indeed, the cytolytic pathways underlying the phenomenon of natural cytotoxicity (3) are an area of intense current investigation in both humans and animal models (6). From such studies, there is increasing evidence that NK cells are an integral component of the host defense system. Not only can these cells confer resistance against certain viral and microbial diseases, but they can also, under certain conditions, mediate strong natural resistance against tumors in vivo.

For example, strains of mice with high NK cell activity have been shown to be more resistant to the growth of NK-sensitive tumor cells than are mouse strains with low NK cell activity (11). Moreover, C57BL beige mice, which have a selective NK cell defect, are more receptive to tumor growth and metastasis than are syngeneic mice with normal NK cell activity (10, 17). A similar condition exists in humans with Chediak-Higashi syndrome, and it has been suggested that the NK cell defect in this disease may be related to the subsequent development of the accelerated lymphoma-like phase observed in these individuals (15). Lymphocytes from chronic lymphocytic leukemia patients, who have a high incidence of secondary cancers (4), have minimal or no detectable NK cell activity (20). In addition, renal allograft recipients, who are treated with high-dose immunosuppressive drugs, have an approximately 100-fold higher risk of developing cancers and also have extremely depressed or abolished NK cell activities (12). Thus, recent evidence from several different approaches supports the possibility of a major role for the NK cell system as an immunosurveillance mechanism against neoplastic cells.

To determine whether natural cell-mediated cytotoxicity may play a more general role as an immunosurveillance mechanism against tumor development, we measured spontaneous cell-mediated cytotoxicity in 79 normal individuals and correlated detailed cytotoxicity data with the incidence of cancer in each individual's family.

MATERIALS AND METHODS

Population Studied. The study group included 79 normal individuals without cancer, aged 22 to 69 years. A detailed family history and medical questionnaire were completed for each individual. To eliminate the possibility of "recall bias," the study was designed so that the interviewer did not know the results of cytotoxicity assays. Fifty-nine persons had a low incidence of familial cancer (2 or less cases among grandparents, parents, or siblings), while 20 had a high incidence of cancer (3 or more cases). The only skin cancer, which contributed toward family cancer incidence, was malignant melanoma (one case). There was no significant difference (p > 0.2) in age between the low (41.3 years) and high (45.6 years) cancer incidence groups.

Cell Line. The cell line K562 was used as target in 51Cr release assays. K562 was derived by Lozzio and Lozzio (13) from a patient with chronic myelogenous leukemia in blast crisis, and was provided to us by Dr. Joyce Zarling (18). These suspension cells upon passing were seeded at 2.5 x 10^6 cells/ml, grown and sustained in culture using Roswell Park Memorial Institute Medium 1640 supplemented with 10% newborn calf serum (Grand Island Biological Co., Grand Island, N. Y.).

Separation of Mononuclear Cells from Peripheral Blood. Approximately 40 ml of peripheral blood were collected in heparinized tubes from each normal adult donor between 8 and 9 a.m. to control for the possibility of a 24-hr circadian rhythm, and each assay included samples from one or more previously well-studied normal individuals. Each blood sample was diluted with one-half volume of HBSS (Grand Island Biological Co.), and mononuclear cells were isolated using Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) gradient centrifugation (1). Diluted blood (35 ml) was underlayed with Ficoll-Hypaque (15 ml), and the gradients were spun at 750 x g for 30 min.

Mononuclear cells were collected and washed 3 times with HBSS and resuspended in 10 ml Roswell Park Memorial Institute Medium 1640 supplemented with 20% human AB serum and 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, collectively referred to as "complete" medium (19).

51Cr Release Assay. Cytotoxicity assays were routinely performed immediately following collection by the method described previously (19), with minor modifications. K562 cells were washed twice with HBSS, then resuspended in complete medium, and counted. Two x 10^5 K562 cells
target cells in 0.20 ml of complete medium were incubated with 250 μCi of \( ^{51} \text{Cr} \) (Na,\( ^{51} \text{CrO}_4 \), New England Nuclear, Boston, Mass.) for 1 hr at 37° and 5% CO\(_2\) in a humidified incubator. The \( ^{51} \text{Cr} \)-labeled target cells were washed 3 times with cold (4°) Roswell Park Memorial Institute Medium 1640 and resuspended in complete medium at a concentration of 1.6 × 10\(^6\) cells/ml.

Labeled target cells (8 × 10\(^3\)) in 50 μl were added to each well of replicate round-bottomed plates (96-well microtest plates; Nunc, Kamstrup, Denmark). Effector cells in 0.2 ml of complete medium were added to the wells at 3 concentrations. Each ratio of effector:target cells (50:1, 25:1, and 6.2:1) was studied in quadruplicate. For determination of spontaneous or maximal \( ^{51} \text{Cr} \) release, 0.2 ml of complete medium alone or detergent (4% aqueous solution of hexadecyltrimethylammonium bromide), respectively, was added to labeled target cells. Plates were agitated on a microshaker for 30 sec and then incubated at 37° and 5% CO\(_2\).

After 3, 4, and 5 hr, plates were removed from the incubator and centrifuged at 165 × g for 5 min. The supernatant was harvested (0.15 ml collected) and counted in a γ-counter. The percentage of specific \( ^{51} \text{Cr} \) released from target cells by the effector cells was calculated using the following:

\[
\text{cpm experimental release} - \text{cpm spontaneous release} \\
\text{cpm maximal release} - \text{cpm spontaneous release} \times 100
\]

Cytotoxicity was also expressed as lytic units/10\(^7\) peripheral blood mononuclear cells. A lytic unit was defined as the number of peripheral blood mononuclear cells needed to effect 55% Cytotoxicity of 1 × 10\(^4\) target cells. Lytic units were determined from cytotoxicity curves, calculated by least squares.

**RESULTS**

Detailed peripheral blood lymphocyte responses of 79 normal individuals were tested against the chronic myeloid leukemia cell line K562. Cytotoxic activity was analyzed from freshly isolated lymphocytes studied in quadruplicate assays after 3, 4, and 5 hr of incubation using 3 different effector:target cell ratios (6.2:1, 25:1, and 50:1). Fifty-nine persons had a low incidence of familial cancer (2 or less cases among grandparents, parents, and siblings), while 20 had a high incidence of cancer (3 or more cases). Table 1 shows that the percentage of \( ^{51} \text{Cr} \) release was significantly lower in each of the 9 separate assay conditions for individuals with a low incidence of cancer compared to individuals with a low incidence of cancer. There was a trend in the significance, as shown in Table 1, with p values from assays with target cell ratios of 6.2:1 > 25:1 > 50:1. Thus, assays with high effector:target cell ratios were apparently more sensitive for the detection of differences in natural cytotoxicity between individuals with high and low incidences of cancer in their families. In addition, the 3- and 5-hr assays were not as sensitive for the detection of these differences as was the 4-hr assay. The relationship between high familial cancer incidence and low natural-cell-mediated cytotoxicity is shown in Chart 1 and was observed in males as well as females and in smokers as well as in nonsmokers (Chart 2). In each of these subgroups, individuals with high incidences of familial cancer had lower natural cell-mediated cytotoxicity.

The same conclusion was reached when the data were expressed in terms of lytic activity with a fixed number of mononuclear cells. For example, as shown in Table 2, the high cancer incidence group had a significantly lower level of lytic units per 10\(^7\) mononuclear cells isolated from Ficoll-Hypaque gradients. This was true for each of the 3 assay conditions, with the 4-hr assay yielding the most significant differences (p < 0.0002). In 41 individuals, peripheral blood levels of circulating lymphocytes were measured simultaneously with the natural cell-mediated cytotoxicity. Again, the high cancer incidence group had a significantly lower level of lytic units per ml of blood (p < 0.01 for 4-hr assay).

Moreover, as shown in Table 3, natural cell-mediated cytotoxicity was inversely correlated with the familial incidence of cancer. The same conclusion was reached using 10 different methods for quantitating familial cancer incidence in our study group.

<table>
<thead>
<tr>
<th>Assay</th>
<th>% of specific ( ^{51} \text{Cr} ) release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hr</td>
<td>Ratios</td>
</tr>
<tr>
<td>3</td>
<td>6.2:1</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
</tr>
<tr>
<td>4</td>
<td>6.2:1</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
</tr>
<tr>
<td>5</td>
<td>6.2:1</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
</tr>
</tbody>
</table>

\( ^a \) Ratio of effector:target cells.  
\( ^b \) Two-sided Student's t test with 77 d.f.  
\( ^c \) Arithmetic mean.
In the current study, we analyzed natural cytotoxicity in a group of normal healthy individuals and then correlated the detailed cytotoxicity data with the incidence of cancer in each individual's family. Significant correlations emerged. In contrast, most previous studies of the putative role of natural cell-mediated cytotoxicity in human neoplasia have focused on individuals following the onset of overt cancer (14). Accordingly, the results of such studies have been inconclusive regarding the relationship between natural cell-mediated cytotoxicity and incidence of neoplasia. Typically, the coexistence of medical therapy, different stages of disease progression at the time of study, and differences in performance status of members of the study group, etc., have made it difficult to extrapolate the relative level of the individual's immunosurveillance capacity against tumor prior to overt disease. A recent study partially addressed this issue by measuring natural cell-mediated cytotoxicity in patients with breast carcinoma at the time of diagnosis and prior to therapy (2). Low cytotoxic activity was observed. While such a study

Importantly, the correlation was significant for all 3 relative groups analyzed, and was independent of family size.

To control for daily variation over time, each assay included samples from one or more previously well-studied normal individuals. By comparing the results obtained from these "control" individuals in a particular assay with their mean result over time, it was possible to establish the stability of the assay over time. When each assay was ranked either as above or below the median, there were equal numbers of individuals with high incidences of family cancer in each group. Further, in each assay, there was the purposeful avoidance of any clustering of individuals from high cancer incidence families in the assay; specifically, in over 80% of the assays, individuals with high familial cancer risk were sex-matched with an individual with low incidence of familial cancer.

No correlation was found between natural cell-mediated cytotoxicity and age. The mean ages of the low and high cancer incidence groups were 41.3 and 45.6 years, respectively. The difference in ages between the 2 groups was not statistically significant (p > 0.2).

**DISCUSSION**

Table 2

<table>
<thead>
<tr>
<th>Assay (hr)</th>
<th>Lytic units/10⁶ cells</th>
<th>Lytic units/10 ml blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>High</td>
<td>p</td>
</tr>
<tr>
<td>3</td>
<td>12.76</td>
<td>8.42</td>
</tr>
<tr>
<td>4</td>
<td>16.48</td>
<td>10.50</td>
</tr>
<tr>
<td>5</td>
<td>19.29</td>
<td>12.97</td>
</tr>
</tbody>
</table>

*a* Two-sided Student's t test with 77 d.f.

*b* Two-sided Student's t test with 39 d.f.

*c* Arithmetic mean.
cannot suggest whether the depressed level was secondary to onset of clinically overt breast carcinoma or preceded it, as if it were involved in the etiological process, it does indicate that the cytotoxicity depression antedates the possible depressive effects of disease progression and the various therapeutic manipulations.

However, our current studies do establish that a lesion in natural cell-mediated cytotoxicity preexists in individuals at risk to the development of cancer who are medically normal at the time of study. Namely, the results herein presented show that individuals with high familial incidences of cancer do have a statistically significant lower natural cell-mediated cytotoxicity than individuals with lower familial histories of cancer. This correlation is seen by a variety of analyses including: (a) comparison of specific 51Cr release data expressed as a percentage of maximum release; (b) comparison of absolute lytic units per 107 mononuclear cells isolated; or (c) comparison of lytic units per ml of peripheral blood. Since the absolute lymphocyte counts of normal individuals can vary by a factor of 3, establishing a level of natural cytotoxicity expressed in terms of circulating blood levels (lytic units per ml) may become a parameter which merits further attention in subsequent studies of natural cytotoxicity, especially in population groups at increased risk of cancer development.

While it has been recognized that certain genetic defects, e.g., Chediak-Higashi syndrome (15), are linked to increased frequencies of cancers and are associated with a profound defect in NK activity, such genetic syndromes can account for only a very small fraction of the tumors seen in the general population. Still, studies such as these support the notion that a number of different genes may control unique steps in the cytolytic pathway which mediate NK activity (5). In familial melanoma, low, natural cell-mediated cytotoxicity has also been detected, both in the patients and in their relatives (7), and it has been argued that this reduction in a specific aspect of immunosurveillance may be an important predisposing factor in the development of recurrent melanoma (8). However, until our current report, there has been a relative paucity of data concerning a possible defect which could contribute to the increased frequency of the more common tumor types seen in the general population.

Thus, it is significant that the 4 most frequent tumor types observed among the family members of our study group (Table 4), namely, breast, colorectal, lung, and prostatic carcinoma, are also the 4 most frequent tumor types associated with cancer deaths in the United States. Moreover, the 5 primary cancers listed, which were the most frequent tumors in family members of our study group, account for the majority of cancer deaths in both males (55%) and females (53%) in the United States as determined by the most recent compilation of the American Cancer Society (16). First-degree relatives in this study were defined as parents and siblings. Second-degree relatives were defined as grandparents and parent’s sibling's.

### Table 3

Correlations between natural cell-mediated cytotoxicity and familial incidence of cancer

In the columns labeled "Numerator," the number of individuals with cancer in each relative group was considered while, in the columns labeled "Ratio," a correction for family size was included, since the number of individuals with cancer in each group was divided by the total number of individuals in that group. In the "Weighted genetically" columns, equal weight was given to a cancer occurring in any of the relative groups, while in the "Unweighted genetically" columns, twice as much weight was given to a cancer present in a parent or sibling of the individual analyzed for NK activity, as compared to a grandparent and parent’s sibling.

<table>
<thead>
<tr>
<th>Relative groups</th>
<th>Weighted genetically</th>
<th>Unweighted genetically</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numerator</td>
<td>Ratio</td>
<td>Numerator</td>
</tr>
<tr>
<td>Parents + Siblings</td>
<td>-0.341 (p &lt; 0.005)</td>
<td>-0.375 (p &lt; 0.001)</td>
</tr>
<tr>
<td>Parents + Siblings + Grandparents</td>
<td>-0.388 (p &lt; 0.001)</td>
<td>-0.385 (p &lt; 0.001)</td>
</tr>
<tr>
<td>Parents + Siblings + Grandparents + Parent’s siblings</td>
<td>-0.338 (p &lt; 0.005)</td>
<td>-0.379 (p &lt; 0.001)</td>
</tr>
</tbody>
</table>

a Numerator = \( \sum w_i \times (\text{No. of individuals in relative Group } i \text{ with cancer}) \)

\[
\text{Ratio} = \frac{\sum w_i \times (\text{No. of individuals in relative Group } i \text{ with cancer})}{(\text{Total no. of individuals in relative Group } i)}
\]

where the summation is over 4 possible relative groups, as indicated in the far left column of the table, and as: (a) Parents, \( i = 1 \); (b) Siblings, \( i = 2 \); (c) Grandparents, \( i = 3 \); and (d) Parent’s siblings, \( i = 4 \); and where \( w_i \) is genetic weighting for Group \( i \). For unweighted genetically:

\[
w_1 = w_2 = w_3 = w_4 = 1
\]

For weighted genetically: \( w_1 = w_2 = \frac{1}{2} \)

and

\[
w_3 = w_4 = \frac{1}{4}
\]

b Values are for the correlation coefficient \( r \), between numerator or ratio as defined above, and for the percentage of specific 51Cr release for 4 hr, 50:1 effectortarget cell ratio; \( p \) values are for the null hypothesis \( p = 0 \), 2-sided alternative, as tested by the Student’s \( t \) test.

c Weighted and unweighted correlation coefficients are identical for relative group (Parents = Siblings), since:

\[
w_1 = w_2 = \frac{1}{2}
\]

### Table 4

Incidence of cancer by site among family members

The 4 most frequent tumor types observed in family members of this study group, namely, breast, colorectal, lung, and prostatic carcinoma, are also the 4 most frequent tumor types associated with cancer deaths in the United States. Moreover, the 5 primary cancers listed, which were the most frequent tumors in family members of our study group, account for the majority of cancer deaths in both males (55%) and females (53%) in the United States as determined by the most recent compilation of the American Cancer Society (16). First-degree relatives in this study were defined as parents and siblings. Second-degree relatives were defined as grandparents and parent’s siblings.

<table>
<thead>
<tr>
<th>Primary Site</th>
<th>First degree</th>
<th>Second degree</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>16</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>Colon-rectum</td>
<td>9</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Lung</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Prostate</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Uterus</td>
<td>1</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>
ulating the incidence of a number of histologically different tumor types which contribute a major fraction of the cancer deaths in the United States.

Our experimental results show an inverse relationship between the familial incidence of cancer and natural cell-mediated cytotoxicity. They support an immunosurveillance function for natural cell-mediated cytotoxicity or NK cell activity, and raise the question as to whether low, natural cell-mediated cytotoxicity may be an important risk factor for the development of a variety of human cancers. The molecular mechanics of natural cell-mediatated cytotoxicity are obviously complex and involve a number of activating factors including interferons, lectins, and various biological substances (6). It will now be important to test the ability of NK cells from patients at risk of cancer development to be augmented by these disparate substances. Such studies may provide further clues to defects in specific gene products. Given the prominent role of interferon in NK cell function (6, 18), defects in the generation (endogenous synthesis) of a particular molecular species of interferon could, for example, contribute to the lowered natural cell-mediated cytotoxicity observed. However, in studies of patients with advanced cancer, the induction of α-(leukocyte) interferon in peripheral blood leukocytes appears to be within normal limits (9). A major contribution of the current study is to suggest that there may be one or more discreet lesions in natural cytotoxicity which are sufficiently common to have impact upon, or be associated with, the pathological processes leading to the major tumor types in our population.

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Low Natural Cytotoxicity of Peripheral Blood Mononuclear Cells in Individuals with High Familial Incidences of Cancer


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