Spontaneous or Natural Killer Cytotoxicity of K562 Erythroleukemic Cells in Normal Patients

James E. Nagel, Gary D. Collins, and William H. Adler

Clinical Immunology Section, Gerontology Research Center, National Institute on Aging, NIH, Baltimore, Maryland 21224

ABSTRACT

Peripheral blood mononuclear cells from 200 normal individuals, ages 20 to 95 years, were evaluated for their capability to mediate spontaneous or natural killer (NK) cytotoxicity using the K562 erythroleukemic cells as targets. The results of a 4-hr ⁶¹Cr specific release assay demonstrated that the NK activity of normal individuals is independent of age, sex, and smoking habits. Although varying greatly among individuals, the NK activity of 25 persons restudied after a mean 20-month interval remained stable. Deficient NK lytic activity is not characteristic of elderly individuals.

INTRODUCTION

The in vitro lysis of tumor cells by lymphocytic effector cells may proceed through the activities of at least 3 types of cytotoxic mononuclear cells: cytotoxic T-lymphocytes; antibody-dependent killer cells; and antibody-independent NK. Among this functional classification, NK putatively have the unique ability to recognize, without prior sensitization, nonantibody-coated targets and to spontaneously lyse these allogenic, syngeneic, and xenogeneic tumor cells. This capability to detect and eliminate nonspecifically foreign cells on initial exposure has led to the proposal that NK function as the primary effectors of immunosurveillance. Although known to be nonphagocytic, nonadherent, and heterogeneous for the presence of Fc receptors for IgG, further morphological characteristics as well as the origin of human NK have not been fully defined. Increasing experimental evidence indicates that both the spontaneous (NK) and antibody-dependent cellular cytotoxic activities of peripheral blood cells are mediated by the same subpopulations, which have been characterized by various investigators to be a T-lymphocyte, promonocyte, or "null" cell or to be of myeloid origin. Many T-cell-mediated immune functions diminish with advancing age; however, the effect of aging on NK activity is unclear. For example, in mice and rats in which the age-related change is most easily demonstrated, the NK activity of spleen cells is absent in the very young, peaks at 5 to 8 weeks of age, and then decreases thereafter. Most studies of human NK activity have indicated that reactivity does not diminish with age; however, many published investigations have dealt with small numbers of individuals or have studied NK activity in ill or tumor-bearing patients of various ages. Despite the lack of conclusive evidence demonstrating an in vivo cause and effect relationship between diminished NK activity and neoplasia, there are sufficient data from the experimental mouse model to support the conclusion that, under certain circumstances, NK are important in resistance to tumor growth. The proposed immunosurveillance role of NK, coupled with recent reports on vital statistics demonstrating that death from malignant neoplasms ranks second to cardiovascular disease as the leading cause of death in individuals over 65 years of age, prompted the present investigation of a large group of well-characterized normal adults on the effect of age on human NK activity.

MATERIALS AND METHODS

Study Population. The study population was composed of 200 adults, ages 20 to 95 years, who are participants in the BLSA. The study group that is reported here included 50 females and 150 males. The BLSA is an investigation of normative human aging which began for males in 1958. A companion program for females was initiated in 1978. The self-recruited participants voluntarily return approximately every 18 to 24 months to undergo a 3-day inpatient reevaluation of psychological, biomedical, and physical changes. Essentially, all participants are ambulatory, and most live in their own residence with a spouse. Stone and Norris have summarized previously the demographic and social characteristics of the male participants. The male subjects included in this report have been reevaluated an average of 8 (range, 2 to 19) times and the female participants have been reevaluated twice. The BLSA program does not make any overt attempts to alter the lifestyle or living habits of the participants, other than to advise the subjects and their personal physicians of any significant medical abnormalities which may be detected during their periodic evaluation.

For the purposes of this study, smokers were defined as those individuals who have a history of smoking at least 10 cigarettes/day. Nonsmokers were considered to be those who were not smoking at the time of their evaluation, but this group does include former smokers.

Isolation of Mononuclear Cells. Heparinized peripheral blood (20 ml) was diluted 1:4 with RPMI Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) and separated by differential buoyancy centrifugation on a Ficoll:sodium diatrizoate cushion (LSM; Litton Bionetics, Kensington, Md.). The interface cells were removed with a Pasteur pipet and washed twice with RPMI Medium 1640, and the mononuclear cell count was determined in a hemocytometer.

Cytotoxicity Assay. Cultured cells from the K562 erythroleukemic cell line (provided by Dr. R. B. Herberman, National Cancer Institute, NIH) grown in RPMI Medium 1640 supplemented with 10% FCS, 10 mM 4-(2-hydroxyethyl)-1-piperazi-
neethanesulfonic acid, 2 mM L-glutamine, and 100 μg genta-
mycin per ml were used as target cells. The line was routinely
subcultured the day before use in an assay. Prior to an assay,
the target cells (2 × 10^6 in 0.15 ml of Tris buffer, pH 7.4) were
incubated with occasional mixing with 0.2 μCi of Naβ51CrO4 in
0.2 ml of sterile isotonic 0.9% NaCl solution (Amerham/Searle
Corp., Arlington Heights, Ill.; specific activity 50 to 100 Ci/mg
chromium) for 30 min at 37°. The chromium-labeled K562
target cells were then centrifuged twice through 2 ml of heat-
chromium) for 30 min at 37°. The chromium-labeled K562
target cells were then centrifuged twice through 2 ml of heat-
inactivated FCS (to remove unincorporated 51Cr), then resus-
pended in RPMI Medium 1640 containing 10% FCS, and
counted. After adjusting the density to 1 × 10^6 cells/ml in
RPMI Medium 1640 with 25 mm 4-(2-hydroxyethyl)-1-piper-
zineethanesulfonic acid and 10% heat-inactivated FCS, 100
μl (10⁴ radio-labeled target cells) were dispensed into each
well of a round-bottom microtiter plate (Linbro Chemical, New
Haven, Conn.) containing 100 μl of the serially diluted effector
cells. Eight target:effector ratios from 1:100 to 1:0.78 were
used. Each ratio was run in duplicate. Following the addition
of the target cells, the microtiter plates were centrifuged for 3 min
at 400 × g and then incubated at 37° in a humidified atmo-
sphere containing 5% CO₂:95% air. After 4-hr incubation, 100
μl of supernatant were removed from each well and placed in
individual 12- x 75-mm glass tubes, and the radioactivity was
determined in a gamma counter (Nuclear Chicago, Des Plaines,
III). The percentage of specific 51Cr release was calculated as

\[
\% \text{ of release} = \frac{E - S}{W - S} \times 100
\]

where \(E\) is cpm in the experimental tube, \(S\) is cpm spontaneous
release, and \(W\) is cpm maximal isotope release effected by
water lysis. Dose-response curves were obtained for each
donor by plotting the percentage of specific 51Cr release
obtained at each of 8 effector:target ratios and the log<sub>10</sub>
of the number of effector cells. The mean spontaneous 51Cr
release by the radiolabeled K562 targets in the absence of effector
cells was 7.1%.

RESULTS

Effect of Age and Gender on NK Activity. The NK activity
of peripheral blood lymphocytes from 200 individuals (50 fe-
male and 150 males) against K562 target cell is shown in
Chart 1. Data analysis by least-squares linear regression of the
percentage of 51Cr release for the entire group demonstrated
no significant differences (at the \(p < 0.05\) level) in NK activity
as a result of advancing age. Since the NK activity of each
individual was tested at 8 effector:target ratios, a comparison
was made of the slopes of the linear portions of the semilog-
arithmetic plot of effector cell number and percentage of
51Cr release. A linear regression model was used to evaluate the
significance of each regression coefficient with those (5 of
200) not meeting the \(p < 0.05\) level of significance being
excluded. Analysis of covariance by age group demonstrated
equality of the regression lines across age groups, indicating
no significant age-related change in peripheral blood NK activ-
ity.

Chart 2 displays a comparison of the peripheral blood NK
activity of males and females according to the age of the cell
donors. There were no significant differences (t test) in periph-
eral blood NK activity of males or females in this investigation.

Because data collection was accomplished over a period of
approximately 2 years, we evaluated our results for the pres-
ence of uncontrolled variables which may have arisen during
the study. This was done by considering the first and last 100
individuals tested as separate groups and subjecting the data
to an analysis of variance test. The results showed that the
population means of each group for both age and NK activity
had \(F\) values of <3.5 and therefore were not statistically
significantly different.

Smoking Status of Cell Donors of Peripheral Blood NK
Activity. Previous reports indicated that NK activity may be
altered by cigarette smoking (7). Of the 200 individuals studied,
41 were found to be active cigarette smokers at the time they
were tested. There were no significant differences found in the
mean peripheral blood NK activity of smokers and nonsmokers.
However, the smoking group had by t-test analysis a slightly
lower mean age (49.3 years) than did the nonsmokers (55.6
years, \(p < 0.05\)), a factor which might obscure differences in
peripheral blood NK activity based on smoking habits.

Longitudinal Follow-up of NK Activity. Chart 3 displays the
results of 2 measurements of peripheral blood NK activity on
the same individuals separated by a 13- to 25- (mean, 19.3)
month interval. Although the group was small \((n = 25)\) and
there was considerable variation among individuals in the levels
of their NK activity, no statistical difference by paired t-test
analysis was found between the first and second determination of peripheral blood NK activity for these individuals.

DISCUSSION

In the present study, the effect of age on levels of human peripheral blood cell NK activity was studied. Using a well-characterized group of 200 normal individuals 20 to 95 years of age, a statistically significant age- or sex-related difference in peripheral blood NK activity against the K562 cell line could not be demonstrated. Although insignificant, it should be noted that cells from individuals between 40 and 80 years of age did manifest decreasing levels of cytotoxic activity. In contrast, cells from individuals over 80 years of age displayed greater levels of mean NK activity as compared with younger age groups. It is important to recognize that this observation is based on cross-sectional analysis of a population and that the older individuals, especially those over 75 years of age, constitute a biologically selected population. These elderly subjects represent the survivors from a group which has already experienced greater than 75% mortality. If in vivo NK activity conveys a protective function via immune surveillance or by an association with other unidentified risk factors, it could be expected that individuals with low NK activity would have been eliminated by death from a population of elderly subjects. The deviation of the downward trend in cytotoxic activity may infer that the elderly subjects represent the more "fit" survivors from a larger group of individuals.

Although a substantial amount of data exists indicating that the NK activity of murine spleen cells declines with advancing age, considerable variation is present among different strains of genetically inbred mice (18). Different patterns of an age-associated decline in NK activity of mice and rats are also observed when different cell types are used as targets (2, 23). For instance, in the rat when carcinoma or sarcoma cells, as opposed to lymphoid cells, are used as targets, constant levels of NK activity are maintained through 22 months of age (2). Other animals, such as the pig, hamster, and guinea pig, although less extensively studied, have not demonstrated an age-related decline in NK activity (44). Prior studies of human NK activity have, in general, demonstrated no age-associated change, although some reports, not using K562 cells as targets, have noted an increase in NK activity with the age of the donor (8, 20, 27, 30, 37, 46, 47). Human peripheral blood NK activity has been reported to be influenced by gender (30, 37), smoking habits (7), histocompatibility haplotypes (31, 40), chronic alcohol consumption (42), and recent viral infection (18); making the singular effect of age on human NK activity difficult to isolate in a cross-sectional study. In addition, individual levels of human NK activity, even among similarly aged normal subjects, are variable. Published information concerning serial measurements of individual cytotoxic activity, in contrast to the finding of the percentage study, has indicated that NK activity of cells from an individual fluctuates considerably (14, 36).

It has also been demonstrated in both animals and humans that NK activity may be augmented by type I interferon or type I interferon inducers (5, 6, 19, 25, 41). Since studies in the mouse have shown previously that advancing cell donor age results is enhanced interferon production by mitogen-stimulated spleen cells, differences in the ability to synthesize interferon may be an important factor influencing or regulating the levels of NK activity of different aged individuals (12). Certainly, interferon is important in the modulation of NK cytotoxic activity; however, the finding of enhanced interferon production in aging mice would appear to contradict the reported age-related decline in their spleen cell NK activity. The variability of the age-dependent decline of NK activity in mice, the peak and persistance of NK activity in older mice (9, 10, 48), and the observation that mice housed in different environments may have greatly different levels of NK activity (3, 28) suggest that the question of the age-related decline in murine NK activity should perhaps undergo a reexamination.

Although identification of cells mediating human peripheral blood NK activity is incomplete, there is general agreement that the cells are nonphagocytic and nonadherent, lack surface membrane immunoglobulin, and bear receptors for sheep erythrocytes and the Fc portion of IgG (reviewed in Refs. 18 and 33). Because of the observation that several of these characteristics are also shared by killer cells mediating antibody-dependent cellular cytotoxicity and because NK and killer cells cannot be physically separated from each other, there has been considerable speculation that both NK and killer cytotoxic activities are mediated by a common cellular subpopulation. Whether these effector cells belong to a population of prethymic T-cells, promonocytes, "null cells", or another cell population remains controversial. Recent data using OKT and OKM hybridoma antibodies and laser flow cytometry suggest that both NK and killer effector cells are not T-cells but are of a myeloid origin (22) and are included in the subpopulation of "null" cells (29).

The relationship between NK activity and primary immunosurveillance is an unproven but interesting hypothesis. Although the cytotoxic NK has many attributes which would allow it to fulfill a role as a primary defense mechanism against small numbers of tumor cells, in vivo evidence of such a role in humans has not and probably cannot be conclusively demonstrated. Most data supporting such a surveillance role for NK have been developed in mice where 3 observations would seem to be the most germane. (a) When tumor cells are transplanted into young and old recipients, there is a lower tumor incidence in young mice with high NK activity than in old
mice with diminished NK activity (11). (b) There is a higher incidence of spontaneous tumors and leukemias among strains of mice with genetically low NK activity compared with strains having high NK activity (48). (c) NK activity may be increased by the injection of tumor cells (16). The possible relationship of these findings to the increased incidence of neoplasia in elderly humans without demonstrably lower levels of NK activity remains speculative. While the present cross-sectional investigation has demonstrated that peripheral blood NK activity did not diminish with age or gender, it is important to consider 2 elements of its experimental design which may be relevant to the results. Since only one cell type, the K562 erythroleukemia cell line, was used as a target, it is important to note that in the mouse model, spleen cell NK activity directed against targets of either solid tumor or lymphoid origin displays different age-related patterns. Another important consideration is that, since our study did not evaluate the NK activity of individuals who were less than 20 years of age, the possibility exists that peak NK activity may occur in younger individuals, and the levels seen in our study group represent decreases from earlier peak activity. This possibility has been suggested by results of other studies (8).

Several additional points should also be emphasized. As suggested by findings from our laboratory and others which indicate that peripheral blood NK activity may be altered by environmental factors, diet, and viral infections, a measurement of the ability to generate an NK cytotoxic response to an appropriate stimulus may be more critical than quantitation of a "basal" level of NK activity in peripheral blood. This would appear, in particular, to be the case if the modulation of NK activity by interferon is found to be related to specific in vivo pathological conditions. Other reports indicating that the augmenting effects of interferon on NK activity occur by recruitment of precursor cells from the bone marrow (38) and that cyclic nucleotides may modulate NK cytosis (34) suggest a variety of other sites at which age-related changes in function could effect NK activity. Studies that compare human NK activities against allogeneic and autologous tumors point out that a critical point in tumor immunosurveillance may be the capacity of the NK effector cell to recognize the tumor cell antigenic determinants (26). Although distinct target structures have been reported on lymphoma cell lines (35), their role in NK-mediated cytosis has been questioned (4). Whether there are alterations in tumor recognition ability that are secondary to age-related changes is unknown (45). An additional investigation of all of these points will be needed to answer the important question of the relevance of NK cytosis to the frequent occurrence of neoplasia in the elderly.

REFERENCES


Spontaneous or Natural Killer Cytotoxicity of K562 Erythroleukemic Cells in Normal Patients

James E. Nagel, Gary D. Collins and William H. Adler