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Relationship between Target Cell Cycle and Susceptibility to Natural Killer Lysis[1]

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ABSTRACT

Studies from several laboratories have evaluated the role of cell surface antigenic molecules on target cells in natural killer (NK)-mediated cytotoxicity. A number of these cell surface molecules are associated with cell proliferation and may be expressed preferentially during one phase of the cell cycle. The purpose of this investigation was to evaluate the role that target cell cycle plays in susceptibility to NK lysis. Enrichment (>80%) of cells from NK-resistant and NK-sensitive cell lines in the G_0G_1, S, and G_2M phases of the cell cycle was achieved by centrifugal elutriation. We demonstrate that there was no influence of cell cycle on NK-mediated lysis of NK-resistant or susceptible cell lines.

INTRODUCTION

NK[3] cells have been documented to spontaneously lyse a variety of target cells including tumor cells (1–4), viral-infected cells (5, 6), and some immature cells of hemopoietic lineages (7, 8). This observation has led to suggestions that the *in vivo* role of NK cells may include immune surveillance against transformed cells, an antiviral effector function, and regulation of hemopoietic cell differentiation (9). A better understanding of the mechanisms used by NK cells to recognize and lyse such a wide spectrum of targets should help to clarify their role *in vivo*.

Targets commonly used to assess NK cell function are leukemic cell lines (*e.g.*, K562 erythroleukemic cells or MOLT-4 T-lymphoblasts) (10). Such target cells are maintained as asynchronous cultures for NK assays. One prominent feature of transformed cells is their tendency to maintain active cell growth and division even under conditions where normal cells cease to proliferate. Despite this biological feature, different tumor cell lines show marked variation in the proportion of cells in different cell cycle phases. Conceivably, such differences could be important in determining the sensitivity of tumor cell lines to NK activity. This would, in turn, suggest that certain target cell receptors mediating steps of NK cell-mediated cytolysis could be expressed in some phases of the cell cycle and not in others. This is the case, for example, for transferrin receptors, whose role in NK cell-mediated cytolysis has been suggested (11).

In the experiments described, we fractionated a variety of NK-resistant and NK-susceptible cell lines by CCE. Subpopulations so obtained were analyzed for their DNA content by PI staining. DNA content analysis demonstrated that subpopulations of cells enriched in each phase of the cell cycle could be obtained, by CCE, from NK-resistant or NK-sensitive tumor cell lines. These cell subpopulations were evaluated for possible differences in susceptibility to NK cell-mediated lysis. The results of these studies support the hypothesis that NK-sensitivity or NK-resistance of target cells is unrelated to cell cycle phases.

MATERIALS AND METHODS

Effector Cells. Heparinized peripheral blood was obtained from normal adult volunteers. MNC were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation (12). MNC were partially depleted of monocytes by 1-h plastic adherence of cell suspensions in RPMI 1640 (Hazelton, CA) with 15% fetal bovine serum (RPMI-FBS). Monocyte-depleted MNC were used as effector cells in the NK cytotoxicity assays.

Target Cells. The erythroleukemia line K562 (13) and KO and CHP-126 neuroblastoma cell lines (14) were used as NK-sensitive target cells. The THP-1-0 monocytic cell line which does bind to NK cells but is refractory to NK lysis was used as an NK-resistant cell (15). Cell lines were maintained in RPMI 1640 supplemented with 5% FBS and 50 μg/ml gentamicin. Cells were in an active growth phase when separated by centrifugal elutriation.

Centrifugal Elutriation. Centrifugal elutriation was performed using a model J6M centrifuge equipped with a JE-6B elutriator rotor housing a Sanderson cell separation chamber (Beckman Instruments, Breg, CA). The procedure for obtaining relatively homogeneous cell populations from the NK-resistant and NK-sensitive target cell lines using centrifugal elutriation was a modification of the long collection method which has been detailed in previous reports (16, 17). Briefly, single-cell suspensions from the various cell lines were elutriated in ice-cold RPMI-FBS. The elutriator system was sterilized using 70% ethanol previous to each elutriator run, and cell separations were performed at 4°C. For each cell line, the rotor speed was reduced 150 rpm at each step following cell loading, and two 40-ml fractions were collected at each step.

For the CHP-126 neuroblastoma cell line, cells were loaded at 3300 rpm, and the rotor speed was subsequently reduced in stepwise fashion. A constant fluid flow rate of 35 ml/min was used throughout the elutriator run.

The THP-1-0, K562, and K0 cells were loaded at a rotor speed of 4000 rpm. The THP-1-0 cells were loaded at a fluid flow rate of 30 ml/min with the flow rate increased to 34 ml/min at the fourth step and to 36 ml/min at the sixth step. The K562 cells were loaded at a flow rate of 31 ml/min with this rate increased to 35 ml/min at the fifth step, and to 39 ml/min at the tenth step. The K0 cells were loaded at a flow rate of 32.5 ml/min with this rate increased to 35 ml/min at the seventh step.

The cells in each fraction were counted, and their cell volume distributions evaluated with an electronic particle counter and channelizer (models ZBI and C1000, respectively; Coulter Electronics, Hialeah, FL). The median cell volume of cells from each fraction was determined with a calibration constant determined previously from studies relating microscopically determined cell size measurements to electronic particle counter settings.

FCM Analysis of Elutriated Fractions. Following elutriation the various cell fractions were stained with PI for DNA content investigations. PI staining was performed as previously described (18). Briefly,

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[3] The abbreviations used are: NK, natural killer cells; CCE, counterflow centrifugal elutriation; PI, propidium iodide; MNC, mononuclear cells; FBS, fetal bovine serum; FCM, flow cytometric.

5×10^5 cells were suspended in 100 μl phosphate buffered saline containing 3% FBS and an additional 100 μl of 100% ethanol added. The cells were fixed overnight and were pelleted at $1500 \times g$ for 1 min. The pellet was incubated with RNase (1 mg/ml; Sigma Chemicals, St. Louis, MO) for 20 min at 37°C. The cells were centrifuged and resuspended in an equivalent volume of a 50 μg/ml solution of PI in phosphate buffered saline. All PI samples were maintained in a light shielded tube at 4°C for at least 1 h before flow cytometric analysis.

The fluorescence of PI stained cells was monitored on an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Immediately prior to FCM analysis, cell suspensions were filtered through a 37-μm nylon mesh filter. PI-stained cells were excited at 488 nm and fluorescence monitored through 515 nm interference and 515 nm long pass filters. Fluorescent microspheres were used to assess instrument performance at the beginning of each run. A minimum of 2×10^4 cells were analyzed for each run.

Cell Cycle Analysis. DNA histogram display and analysis was performed on a Terak 8600 minicomputer (Terak Corp., Scottsdale, AZ), using software developed by Salzman *et al.* (19) and extensively modified by Robinson and Leary of the University of Rochester. The "Simple Fit" program of Dean (20) as adapted to the Terak 8600 (21) was used to estimate the distribution of cells within cell cycle compartments. This software uses a second-order polynomial to mathematically define the S-phase region of the cell cycle, with the remaining cells assigned to the G_0/G_1 and G_2M regions.

Single Cell Cytotoxicity Assay. Effector cells ($2, 1,$ and 0.5×10^6) were admixed with the various unseparated or elutriated fractions (5×10^4) (effector to target ratios, 40:1, 20:1, and 10:1), and centrifuged at $200 \times g$ for 7 min. Cell pellets were incubated for 10 min at 37°C in a water bath to allow conjugate formation and gently resuspended in RPMI-FCS, at a concentration of 5×10^4 cells/ml. Previous studies have shown that recycling of NK cells does not occur under these conditions (22). Cell suspensions were incubated at 37°C for 4 h, centrifuged, and cell pellets stained with propidium iodide and evaluated by FCM to assess cell death (23). Unfractionated or elutriated fractions of target cells were incubated alone to assess spontaneous cell death, and served as controls.

RESULTS

Enrichment of Cells into G_0G_1, S, and G_2M in Fractions by Centrifugal Elutriation. The NK-susceptible cell lines K562, KO, and CHP-126 and the NK-resistant cell line THP-1-0 were separated by CCE. The sizing of cells in each fraction was performed using a Coulter channelizer (Fig. 1) and position in the cell cycle determined by propidium iodide staining. The results of this cell separation procedure showed a greater than 80% enrichment for G_0G_1, S, and G_2M could be obtained in each of the CCE fractions obtained from all of the cell lines tested (Fig. 2).

NK Susceptibility Is Unrelated to Cell Cycle. Each of the cell lines separated by centrifugal elutriation was tested in a 4-h single cell cytotoxicity assay. The K562 target cell, which is the classic cell line used to assess NK cell-mediated cytotoxicity, showed no difference in NK susceptibility for the various CCE fractions predominantly comprised of cells in each phase of the cell cycle (Table 1). Similar results were obtained using two neuroblastoma lines that are sensitive to NK-mediated lysis (KO and CHP-126, Table 1). Thus each fraction predominantly comprised of cells in a distinct phase of the cell cycle showed the same NK sensitivity as the unfractionated population. We also performed experiments to determine if an NK-resistant tumor line might display transient NK sensitivity during one phase of the cell cycle. To address this question the THP-1-0 monocytic line which is resistant to NK lysis was utilized. The results of these experiments (Table 1) show that by enriching for cells in each phase of the cell cycle we could not demonstrate any change in resistance to NK-mediated lysis.

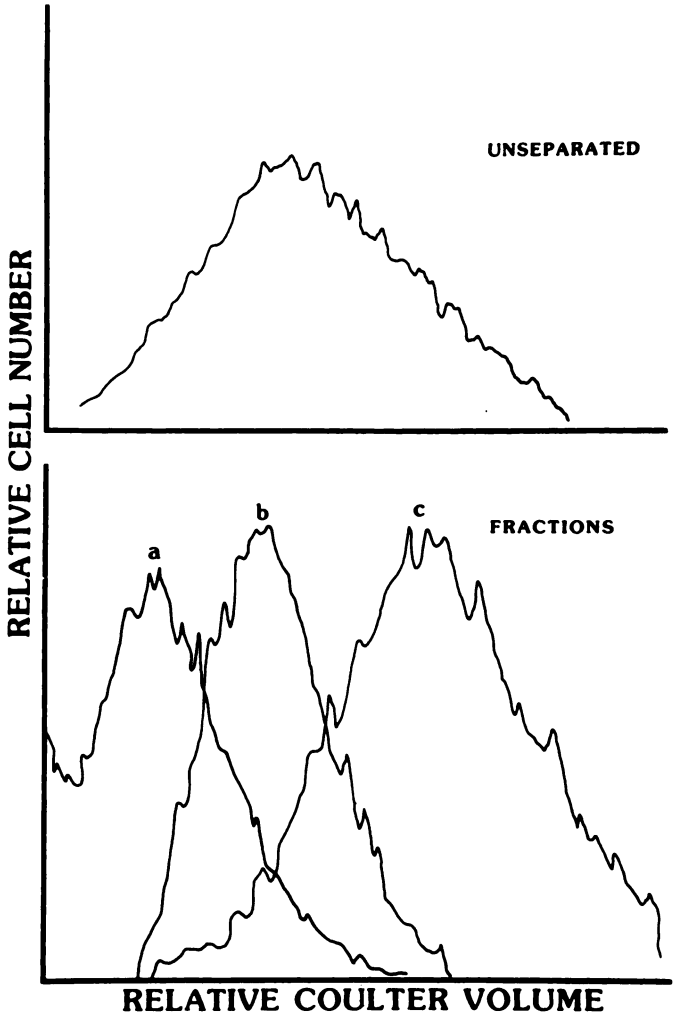

Fig. 1. Representative cell volume profiles of asynchronously growing K562 cells. *Top panel*, total (nonelutriated) cells; *bottom panel*, result obtained from three fractions following cell separation by centrifugal elutriation. Fractions with increasing cell volume (*a*, *b*, *c*) correspond to G_1/G_0, S, and G_2M-enriched cells, respectively, as displayed in Fig. 2, *B–D*.

DISCUSSION

We have evaluated NK cell-mediated cytotoxicity with susceptible and resistant targets following fractionation by counterflow centrifugal elutriation. By enriching for cells in the various phases of the cell cycle (G_0G_1, S, and G_2M), we were able to show no significant differences in NK-mediated cytotoxicity. Thus these results argue against the dependence of NK cell-mediated cytotoxicity on target cell cycle. It is possible that due to incomplete synchronization (*i.e.*, <100% enrichment in the particular cell cycle phases) a subtle difference in NK cytotoxicity as a function of target cell cycle could be present.

Changes in shape, size, and density of cells occur during the cell cycle. Cells which grow as adherent monolayers detach from the substrate during mitosis and can be recovered as relatively purified fractions using mitotic selection (24). While this method provides excellent synchronization for evaluation of cells in M and G_0/G_1, cell progression is required to obtain synchronous populations at later times in the cell cycle. Variations in cell cycle transit generally result in significant synchrony dispersion by the time the cohort of cells enters late S- and G_2 phases. CCE provides a better way of separating cells in various phases of the cell cycle as also shown by this study. CCE is also applicable for cells growing in suspension (*e.g.*,

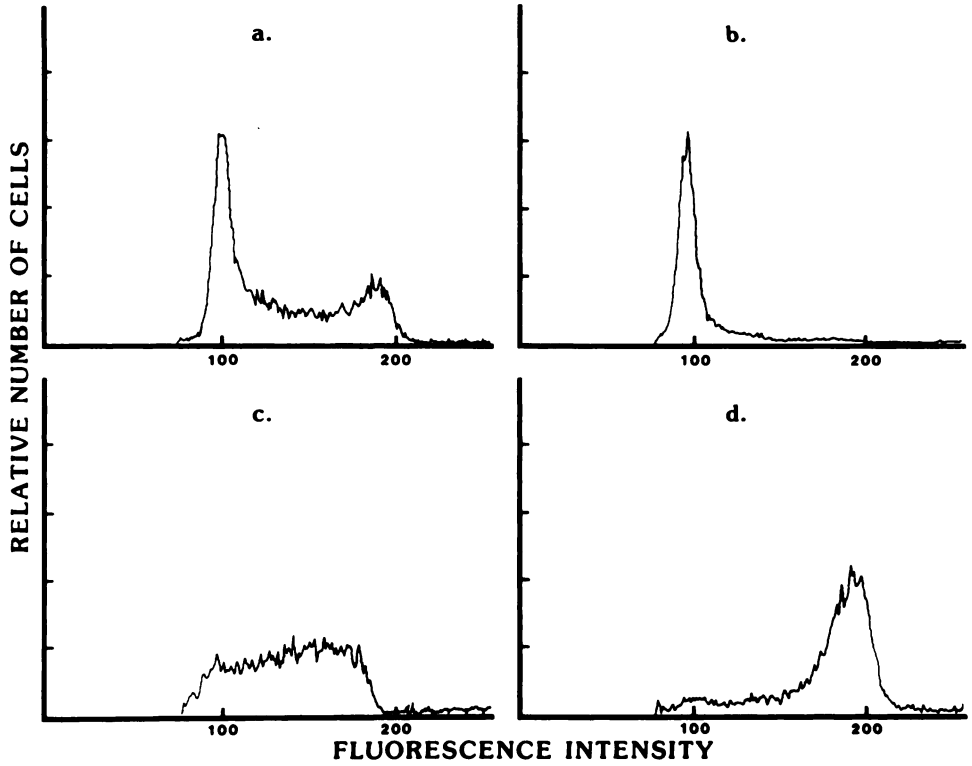

Fig. 2. DNA content histograms of K562 erythroleukemia cells obtained following flow cytometric analysis. Following separation by centrifugal elutriation of asynchronously growing K562 cells each fraction was stained and analyzed for DNA content as described under "Materials and Methods." *A*, unseparated cells (40% G_0G_1, 39% S, and 21% G_2M); *B*, G_0G_1 enriched fraction (>80%); *C*, S enriched (>80%); *D*, G_2M enriched (>80%).

Table 1 *NK cell susceptibility is unrelated to cell cycle phase of target cell*

Natural killer activity was assessed in a propidium iodide dye exclusion and flow cytometry 4-h single cell assay. Data is expressed as lytic units/10^7 cells. One lytic unit equals the number of effector cells required to yield 20% specific lysis.

	Target			
Cell cycle phase	K562	CHP-126	K0	THP-1-0
Unfractionated	54 ± 6[a]	18 ± 3	19 ± 4	<1
G_0-G_1	56 ± 4	15 ± 2	16 ± 3	<1
S	54 ± 3	16 ± 2	17 ± 2	<1
G_2-M	53 ± 2	17 ± 3	18 ± 2	<1

[a] Mean ± SD of three separate experiments.

K562), and in adherent monolayers (CHP-126 and KO cell lines).

Studies from a number of laboratories have examined possible target cell-dependent mechanisms involved in NK-mediated lysis. These studies have evaluated a number of potential recognition structures including major histocompatibility complex antigens (25), cell surface glycolipids and glycoproteins (26), and lectin-like receptors recognizing sugar moieties (27). Other investigators have evaluated the differentiation state of NK targets (28) and have found conflicting results with regard to NK sensitivity following differentiation. Other proposed target molecules for NK cells are these associated with cell proliferation. One such molecule is the transferrin receptor (11). The results of the current study would suggest that cell surface molecules associated with proliferation are not involved in NK recognition events. Recently Lattime *et al.* (29) showed similar results with murine NK cells. They demonstrated that both the kinetics and magnitude of natural killer and natural cytotoxic cell lysis were unaffected by the stage of the target cell cycle.

Thus, our studies extend the observation that human NK cell susceptibility of a variety of targets is independent of cell cycle phase. These studies indicate that differences in the major cell cycle phases associated with active cell proliferation (*i.e.*, G_0G_1, S, and G_2M) do not have a major role in influencing NK sensitivity. However, current data suggest that most cells in many *in vivo* tumor systems are in a resting (*i.e.*, G_0) state. The relative "sensitivity" of G_0 as opposed to G_1 phase cells to NK activity remains unanswered at the present time. Studies to test this hypothesis would include use of techniques such as acridine orange staining which discriminate the G_0 from G_1 phase followed by cell separation using flow cytometry.

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