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# Purification and characterization of cytolytic and noncytolytic human natural killer cell subsets

(cell cycle/conjugates/flow cytometry/phenotype/recycling)

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**ABSTRACT** **Natural killer (NK) cells form three functionally distinct populations of effectors: competent cytolytic effectors able to bind and kill target cells and two subsets of nonlytic effectors, one able and the other unable to bind target cells. A flow cytometric method was developed, based on size and two-color fluorescence of NK cell–target conjugates, for the characterization and sorting of highly purified subpopulations—killer cells, nonkiller binder cells, and free cells. Ultrastructural examination revealed that granule content was reduced in the killer cells and absent in most of the binder cells. Quantitative differences in the expression level of HLA class I, CD11b (C3bi receptor), and CD16 (receptor for the Fc portion of IgG) antigens could differentiate the subsets. The killer phenotype was HLA$^{lo}$, CD11b$^{very\ hi}$, and CD16$^{very\ lo}$; the binder phenotype was CD11b$^{hi}$ and CD16$^{lo}$; and the free-cell phenotype was CD11b$^{lo}$ and CD16$^{hi}$. Cell activation was not requisite for lytic function because no difference in either expression of activation markers or cell cycle could be established among the sorted subpopulations. Although recycling function was inhibited, retention of lytic activity was enriched 4-fold in the sorted killer cell population. These results represent characterization of a successful bulk isolation of competent killer, nonkiller binder, and free cells in human NK-cell populations and should aid our understanding of NK-cell development, lineage, and function.**

When purified or clonal populations of cytolytic effector cells are used in cell-mediated cytotoxic (CMC) reactions against susceptible target cells, only a subset are competent killer cells (1–4). Analysis at the single-cell level demonstrates that cytolytic cell populations consist of three functionally diverse subsets: (*i*) cells that bind and kill target cells, (*ii*) cells that bind but do not kill target cells, and (*iii*) cells that neither bind nor kill their targets. However, to date, ascribing a specific phenotype to cytolytic capacity or stage of differentiation has not been possible. Absence of such markers has precluded the isolation of functional natural killer (NK) subpopulations; thus, the basis for functional heterogeneity is not known.

In a previous study (5), cytometric analysis was used to identify competent cytolytic conjugate-forming cells, nonlytic conjugate-forming cells, and nonconjugate-forming cells in a CMC reaction. In this study, human NK-cell subsets have been sorted and examined for their phenotypic and functional properties.

## MATERIALS AND METHODS

**Monoclonal Antibodies (mAbs).** NK receptor for the Fc portion of IgG (CD16)-specific mAbs B73.1 (IgG1) and 3G8 (IgG1) were gifts from G. Trinchieri (Wistar Institute, Philadelphia) (6, 7). LFA-1 (CD11b)-specific mAb TS.1/22.1.1.1, IgG1 (8) was provided by T. Springer (Harvard Medical School). Anti-transferrin receptor-specific mAb, OKT9, IgG1, was provided by S. H. Golub (University of California, Los Angeles). OKM-1, IgG2b (CRL 8026) specific for CD11a; OKT3, IgG2a (CRL 8001) specific for CD3; OKT8, IgG2a (CRL 8014) specific for CD8; PA2.6, IgG (HB 118) specific for HLA-A,B,C, monomorphic determinants; and L227, IgG1 (HB 96) specific for human Ia determinants (American Type Culture Collection) were used in ascites form at a final dilution of 1:1000. Murine IgG1 isotype control, NK-specific anti-Leu 19, IgG1 (CD56) (9), and anti-Leu-7 (CD57), IgM, and activation antigen-specific anti-Leu 23, IgG1 (10) were purchased as phycoerythrin (PE) conjugates from Becton Dickinson. PE-conjugated F(ab')$_2$ goat anti-mouse IgG (PE–GAM) (Tago) was used as a secondary reagent when needed.

**NK-Cell Enrichment.** Human peripheral blood was centrifuged on Ficoll/Hypaque, and buffy layers were harvested, washed, and resuspended in culture medium [5% bovine calf serum (Irvine Scientific)/RPMI 1640 medium (GIBCO)]. Nonadherent human peripheral blood lymphocytes, used for fluorescein isothiocyanate (FITC) labeling, were obtained by sequential incubations on plastic tissue-culture dishes and then on prewarmed, washed nylon-wool columns, each for 1 hr at 37°C in 5% $CO_2$/air. Aminoethylisothiouronium bromide-sheep erythrocytes were prepared as described (11), and FITC-labeled peripheral blood lymphocytes were rosetted as in ref. 12 at 24°C. Triggering through the CD2 molecule is not favored under these conditions. NK-enriched, rosette-negative cells were routinely 85% ± 10% OKM-1$^+$ (CD11b), 75% ± 10% B73.1$^+$ (CD16) and Leu19$^+$ (CD56), and 8% ± 5% OKT3$^+$ (CD3).

**FITC Labeling.** FITC (Sigma) was dissolved in dimethyl sulfoxide to a concentration of 50 mM. Dissolved FITC was diluted 1:1000 in phosphate-buffered saline (PBS), pH 8.0, and filter sterilized. Human peripheral blood lymphocytes were washed twice without serum and gradually diluted to a concentration of 1–5 × $10^7$ cells per ml in 50 μM FITC, pH 8.0/PBS, incubated 10 min at 37°C, washed three times with PBS, pH 7.0, and resuspended at 2 × $10^7$ cells per ml of culture medium for subsequent NK-cell enrichment. FITC-treated cells and untreated cells showed no difference in NK activity when tested in a $^{51}$Cr-release assay.

**NK-Cell Conjugation and CMC Assay.** NK-enriched cells (5 × $10^6$ cells per ml) were added to K-562 target cells ($10^7$ cells per ml) at an effector-to-target ratio of 1:2, favoring the formation of single effector-bound target conjugates (13). Conditions for CMC and preparation of samples for flow cytometric analysis were followed as described (5). CMC reactions were constantly resuspended by gentle agitation during the 37°C incubation. Samples were placed on ice, and

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Abbreviations: CMC, cell-mediated cytotoxicity; FITC, fluorescein isothiocyanate; HLA$^+$, HLA-positive expression equal to control levels; mAb, monoclonal antibody; NK, natural killer; PE, phycoerythrin; PI, propidium iodide.

propidium iodide (PI, 4 μM final concentration; Sigma) was added before analysis.

**Flow Cytometric Analysis and Sorting of NK-CMC Subpopulations.** An EPICS C (Coulter) flow cytometer was used for analysis and cell sorting. Two-parameter analysis of forward-angle light scatter, a size parameter, and green fluorescence enabled identification of the unconjugated free cells and NK-target conjugates by quadrant analysis. Killer cells bound to targets undergoing death were detected by measuring the red fluorescence, indicating uptake of the vitally excluded dye PI within the gated conjugate population. Sorted free cells routinely contained <3% K-562 cells, a fact suggesting that the level of contamination in the other sorted cell populations was also very low. Sorted cell populations were held overnight at 4°C for subsequent analysis.

**Phenotyping Sorted Cell Populations.** Specific mAbs were added to at least $1 \times 10^4$ cells in 50 μl of cold Hanks' balanced salt solution/0.5% bovine serum albumin and incubated for 45 min on ice. Cells were washed twice and stained with a 1:50 dilution of PE–GAM. After 30 min cells were washed twice and fixed in 1% paraformaldehyde/0.1% sodium azide/PBS solution. Phenotypic analysis was done on cells gated for FITC and small size to exclude analysis of K-562 cells.

**Cell Cycle Analysis.** Control and sorted cell populations were fixed in 50% (vol/vol) ethanol/PBS, washed twice in PBS, and treated with 0.1% RNase (Sigma) for 30 min at 37°C as described (14). Samples were washed twice with PBS and stained with PI (40 μg/ml; Sigma).

**EM.** In brief, sorted and control samples were crosslinked with 2% (vol/vol) glutaraldehyde, postfixed with OsO4, then dehydrated with ethanol, and embedded in Spurr (Pelco, Redding, CA) (15). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100CX electron microscope. Giemsa-stained cytospin preparations were done to confirm the EM data; however, this method was not optimal for detecting different levels of granules on a per-cell basis.

**Single-Cell Assay in Agarose.** To test recycling activity, control 4-hr CMC mixed and sorted populations of binder and killer cells were incubated at 37°C overnight in 5% calf serum/RPMI 1640 medium without added lymphokines, placed over Ficoll/Hypaque to remove dead cells, repelleted with fresh K-562 target cells, and tested in a single-cell assay in agarose as described (16). The absolute-killer frequency refers to percent of killer cells in the total NK population and is derived by extrapolating data using the equation: (% binder × % killer) ÷ 100.

**Statistical Analysis.** The paired Student's *t* test, which uses control populations in parallel with experimental conditions, was applied to test the significance of the phenotype data ($n = 5$ experiments). The $\chi^2$ test was applied to data from EM tabulations and single-cell assay experiments from one representative experiment to derive *P* values.

## RESULTS

**Determination of NK Binder- and Killer-Cell Frequencies by Using Flow Cytometric Analysis.** Fig. 1*A* presents two-parameter displays of logarithmic values of forward-angle light scatter, a size parameter, and of green fluorescence, characterizing cell populations found 15 min into a NK-CMC reaction, control NK-enriched populations, and K-562 populations. Enumeration of active killer cells in the gated conjugate fraction and dead cells within the gated NK-enriched cells or K-562 target cells detected at 1 hr of NK-CMC is displayed in Fig. 1*B*. Fig. 1*C* displays mean percentages of both total conjugates and conjugates containing PI, as well as the mean total or absolute number of killer cells from cytometric data derived from five separate kinetic studies. These data show that at the start of the assay 30–40% of enriched NK effector populations bind K-562 target cells. With time, the conjugate fraction dissociates rapidly and is maintained at 25–50% of the original conjugate frequency for at least 8 hr. These results indicate that NK nonkiller conjugates do not dissociate from their target cell. The percent of PI-positive conjugates increases slightly with time and declines at the end of the assay period.

It was necessary to adhere to a sorting schedule to achieve homogeneous sorted populations. Free cells were sorted only at the beginning of the assay to exclude dissociated killer cells. Because binder cells remain bound to their target cell, they were sorted only at the end of the assay to reduce the possibility of including "late" killer cells. And, because the

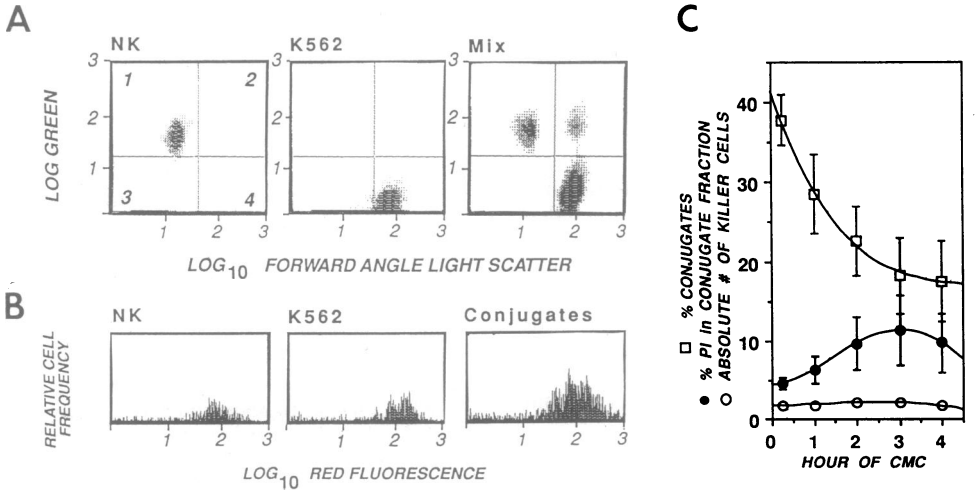


FIG. 1. FITC-labeled, NK-enriched cells pelleted with K-562 targets were gently resuspended before prolonged incubation at 37°C in a shaker bath. Aliquots of cells were taken at the indicated times, and PI was added to the sample (4 μg/ml, final concentration) just before analysis. (*A*) Two-parameter profiles of forward-angle light scatter and green fluorescence are shown at 15 min for control NK and K-562 populations as well as for NK/K-562 mixed CMC sample. One hundred thousand events are displayed at threshold levels of 4, 16, and 32. Quadrants: 1, free FITC-labeled NK cells; 4, free unstained large K-562 target cells; 2, large fluorescent events representing NK–K-562 target-cell conjugates. (*B*) Single-parameter distributions of PI uptake within the indicated gated populations identified in a NK-CMC preparation at 1 hr. Twenty thousand conjugates were analyzed. PI-positive values for the gated populations are as follows: NK, 2.6%; K-562, 2.3%; conjugates, 8.4%. (*C*) Conjugate dissociation and killer-cell detection kinetics during NK-CMC. Mean and SEM values derived from flow cytometric analysis of five separate experiments are shown.

absolute killer-cell frequency did not appreciably change at any time throughout the CMC assay period, killer cells were continuously sorted from as early as 15 min to the end of the assay.

**Ultrastructural Studies on Killer, Binder, and Free Sorted Populations.** The relationship of granularity to lytic function was determined by examining the sorted populations with EM. Heterogeneous cell structure, with respect to granular content, was evident in all populations examined, varying between high—greater than four electron-dense granules per cell, low—with less than four granules per cell, to none (Fig. 2). Tabulation of these groups revealed that a distinctive reproducible profile could be attributed to each population. Table 1 represents data from one of three similar experiments. Effector cells found in the killer fraction retained 75% granularity after delivery of the lethal hit. However, the frequency of cells with a low-granular phenotype was significantly increased over unsorted control-cell levels ($P \leq 0.001$) and suggests that partial degranulation occurred. A striking paucity of electron-dense granules was seen in most cells examined from the sorted binder population. Agranular cells comprised 59% ($P \leq 0.001$) of the binder subset, and highly granular cells represented only 22% of the population versus >40% found in the control and other sorted populations ($P \leq 0.001$). Noncytolytic free cells possessed a granularity distribution identical to the control NK population. These results show that granule content is important for killing function within conjugate-forming NK cells because killer cells are enriched for granular cells, whereas binder cells are not. However, granularity may be necessary but is not sufficient for lytic function.

**Phenotypic Profile of Sorted NK Subpopulations.** Fig. 3 displays phenotype profiles completed on sorted NK-CMC subsets and unsorted control populations of NK-enriched cells and NK-enriched cells mixed with K-562 cells. When compared to parallel control NK cells, sorted killer cells were reduced in both the percentage of HLA-A,B,C ($P \leq 0.05$) and CD16 ($P \leq 0.1$)-bearing cells and in the mean channel intensity of these markers ($P \leq 0.05$ and $P \leq 0.01$, respectively). Only a reduced mean channel intensity was observed in the sorted binder cells for CD16 ($P \leq 0.01$). An identical staining pattern was seen by using 3G8, a mAb specific for a different CD16 epitope (data not shown). CD11b mean channel intensity values varied in all groups: lower expression in sorted free cells and higher expression for sorted binder cells and killer cells ($P \leq 0.1$, 0.1, and 0.025, respectively). In addition, CD11b mean channel intensities were significantly different between binder cells and killer cells ($P \leq 0.05$). It is apparent that CMC reactions induce an increase of CD11b expression because mean channel values are higher in the mix control population than in control NK levels. No significant differences in phenotype were detected among the sorted populations by using antibodies to other NK-specific antigens, CD56 and CD57, as well as to other antigens with overlapping specificities: CD8, CD11a, CD29, and CD45R or to T-cell-specific CD3. No significant enrichment of activation markers, such as HLA class II, transferrin receptor (CD71), interleukin 2 receptor (TAC, CD25), or Leu 23 (CD69) was detected on cells kept on ice immediately after the sorting procedure (Fig. 3 and data not shown).

These data describe phenotypic profiles unique to each sorted population. Relative to the input NK-enriched cells, possessing the phenotype $HLA^+$ (HLA-positive expression equal to control levels), $CD11b^+$, $CD16^+$; free cells are $HLA^+$, $CD11b^{lo}$, and $CD16^{hi}$; binder cells are $HLA^+$, $CD11b^{hi}$, and $CD16^{lo}$; and killer cells are $HLA^{lo}$, $CD11b^{very\ hi}$, and

Table 1. EM analysis of sorted populations

| Sample | Granular, % High* (P) | Granular, % Low† (P) | Granular, % Total‡ (P) | Agranular, % (P) |
|---|---|---|---|---|
| NK | 41.2 ± 2.1 | 17.6 ± 1.6 | 58.8 ± 3.7 | 41.2 ± 3.6 |
| Free | 40.3 ± 5.2 (NS) | 18.6 ± 9.6 (NS) | 58.9 ± 5.0 (NS) | 41.1 ± 5.0 (NS) |
| Binder | 21.8 ± 10.0 (≤0.001) | 18.7 ± 2.8 (NS) | 40.5 ± 8.2 (≤0.001) | 59.5 ± 8.5 (≤0.001) |
| Killer | 43.1 ± 4.9 (NS) | 33.1 ± 7.2 (≤0.001) | 76.3 ± 2.3 (≤0.001) | 23.7 ± 2.3 (≤0.001) |

Grids of embedded sorted samples were prepared for viewing by EM. Triplicate fields, using two separate grids, were evaluated from one representative experiment. More than 35 cells per field (on average, 50 cells per field) were counted for derived percentages. *P* values (in parentheses) are derived from $\chi^2$ test analysis comparing data from sorted populations to data from the control NK sample. NS, not significant.

*More than four electron-dense granules (black as opposed to gray) per cell.

†Less than four electron-dense granules per cell.

‡Sum percentage of high- and low-granule-containing cells.

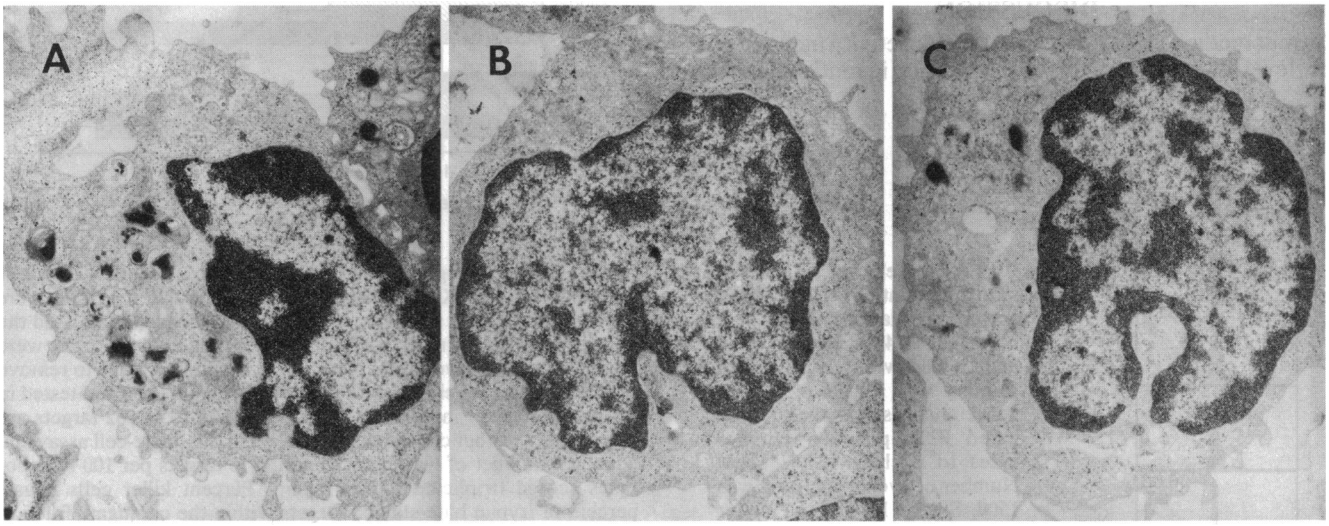


FIG. 2. EM of sorted populations. (*A*) Free cell representing a highly granular cell. (*B*) Binder cell representing an agranular phenotype. (*C*) Killer cell has the low-granular phenotype—i.e., less than four electron-dense granules per cell. Electron-diffuse areas (gray as opposed to black) were not tallied as granules for values derived in Table 1. (Magnification $\times 10^4$.)

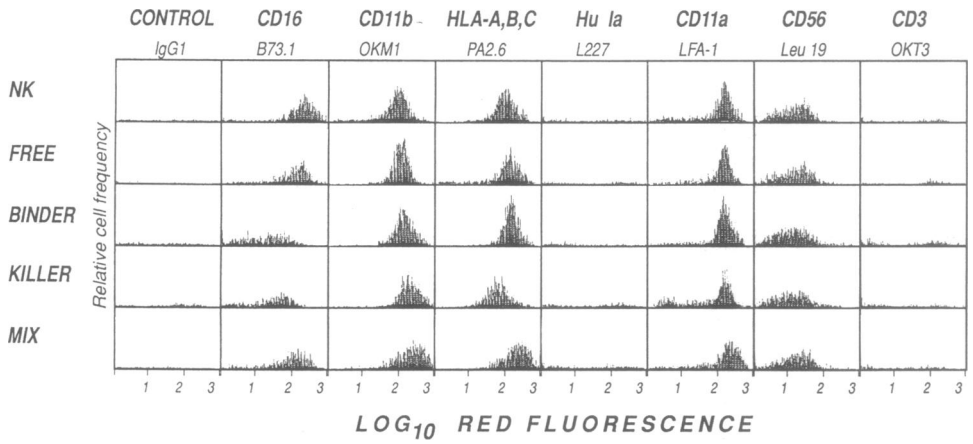


FIG. 3. Phenotype profile of sorted NK populations. Control and sorted populations of cells were kept overnight at 4°C before the staining procedure. Single parameter displays of red fluorescence show 1500 events. NK, NK-enriched cells; MIX, NK-enriched cells mixed with K-562 cells.

$CD16^{very\ lo}$ with respect to mean channel intensity and, for HLA and CD16, percent positivity as well.

**Cell Cycle Analysis.** Measurement of DNA content demonstrates that fresh NK-enriched cells are resting in the $G_0/G_1$ stage, as shown in Fig. 4. Although a minor population of cells in S, $G_2$, and/or M stage could be detected in all populations, no appreciable enrichment of cells in these phases was seen in any of the sorted populations. Therefore, the ability or inability to bind or kill cannot be attributed to a particular stage of the cell cycle.

**Recycling Function of Killer and Binder Populations.** After the sorting procedure, cells were incubated at 37°C overnight and were then tested for ability to recycle—that is, to undergo second-round binding and killing of fresh targets in the single-cell assay (Fig. 5). Both unsorted (referred to as mix) and sorted CMC cell populations lose >50% of their binding as compared with unsorted control NK-enriched cells (data not shown) and original binding frequency, respectively. Lytic capacity within conjugates was also reduced in unsorted and sorted killer samples. However, some killing activity was evident in the binder cell sorted population. Whether lytic binder cells represented contaminant killer cells or whether they developed activity overnight was not ascertained. Free cells maintained a low binding and killing frequency when compared with controls (data not shown). Overall, these results show that NK cells are poor in recycling function but that some function is retained. Significantly, lytic function is enriched 4-fold over controls in the sorted killer population.

## DISCUSSION

Current methods measure cytotoxic cell activity indirectly at the level of target-cell lysis. The direct identification and enumeration of competent killer cells has relied on the single-cell assay in agarose (16). This method, however, is limited by reliance on subjective data acquisition; more importantly, the subpopulations cannot be retrieved for further characterization. Flow cytometric analysis has previously been applied for indirectly measuring cytolytic cell activity (17–20) and to directly identify killer cells actively lysing their bound targets (5). The present study successfully isolates and subsequently characterizes competent killer cells as well as nonlytic binder and free cell populations.

The correlation of granule content and cytolytic capacity within NK cells has been well documented (21–27). However, it is unclear how much degranulation is sufficient for lysis and, for that matter, to what extent other nongranular mechanisms contribute. We found that most sorted killer cells were granular but were of the low-granular phenotype compared with controls. Additionally, in view of our findings that free cells contained control levels of granularity, granule content is not a sufficient marker for specific identification of killer cells. The absence of granules in most binder cells suggests that acquisition of binding occurs before development of granular lytic machinery. Alternatively, binder cells

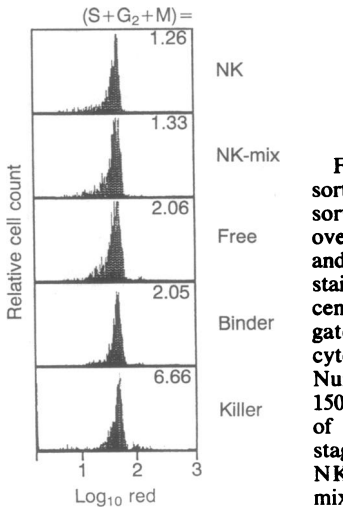


FIG. 4. Cell cycle analysis of sorted populations. Control and sorted populations were kept overnight at 4°C and then fixed and treated with RNase before staining with PI. Red fluorescence analysis was analyzed on gated FITC-positive lymphocytes to exclude K-562 cells. Number of events analyzed was 1500. $S+G_2+M$, additive count of cells in S, $G_2$, and M cell stages; NK, NK-enriched cells; NK-mix, NK-enriched cells mixed with K-562 cells.

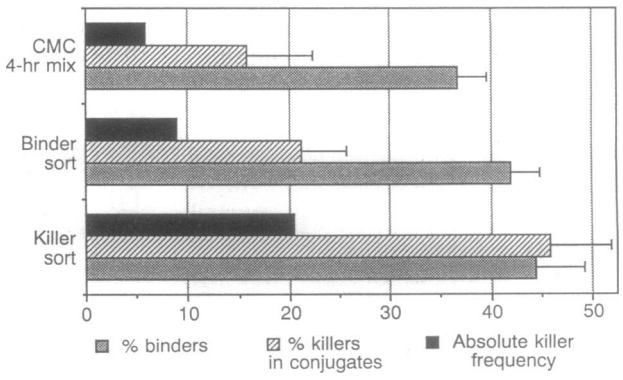


FIG. 5. Recycling function of sorted binder and killer cells. NK cells, pelleted with K-562 cells and incubated for 4 hr for CMC, were used as a source for sorted populations (binders and killers) and the control population (mix). To test for recycling activity, cells were incubated at 37°C overnight, placed over Ficoll/Hypaque to remove dead cells, then repelleted with fresh K-562 target cells and tested in a single-cell assay in agarose. NK cells bound to K-562 targets are referred to as either conjugates or binders in the single-cell assay and equal the number of lymphocytes bound to targets per 100 lymphocytes scored (triplicate scores taken). Percent killer cells equals percent of trypan blue-stained targets within the conjugate fraction (>50 conjugates, scored in triplicate). *P* values are ≤0.0001 and ≤0.005 for binder and killer cells, respectively, for binding capacity and ≤0.0001 for killer cells in lytic capacity. All other *P* values were not significant.

may represent a mature sublineage involved in nonlytic regulatory functions.

Previous studies (28, 29) attempted to define a killer cell-associated marker within the $CD16^+$ NK-cell population by first sorting and then testing function but did not succeed. We hoped that by sorting functionally defined NK subsets first, subsequent phenotypic analysis might reveal a specific marker or profile that correlated with function. By using a large panel of mAbs specific for a number of markers, we failed to identify specific molecules exclusively associated with any of the sorted cell subsets. However, we could distinguish between sorted killer cells, binder cells, and free cells by the relative expression of HLA class I, CD11b, and CD16 molecules. These molecules, therefore, may be involved in the lytic pathway in either recognition or signaling or be modulated after effector–target binding for other unknown functions.

Our results indicate that expression of HLA class I is lower and CD11b expression is higher on sorted killer cells when compared with controls. Increased CD11b expression was induced by target interaction, and high expression can enrich for killer cells present in fresh NK cells (unpublished observation). A previous report (30) suggests a possible role for NK class I molecules in the lytic pathway. In addition, increased CD11b expression seen on killers is corroborated by previous studies on effects of NK–target interactions (12). However, the reason for differential expression of these surface markers is not yet understood.

Killer cells must start as $CD16^+$ cells because lytic activity has been shown to reside only in the $CD16^+$ fraction of fresh NK cells (29, 31). Previous studies indicate that NK and antibody-dependent cell-mediated cytotoxic activities are mediated by separate receptors (31, 32). Because of these findings, we were surprised that interaction with NK targets partially down-modulated surface CD16. The CD16 molecule is known to be down-modulated easily by specific crosslinking of the receptor for the Fc portion of IgG (33). Our results show that NK function can also affect CD16 expression; the nature of this link remains to be investigated.

Whether killer cells, present in peripheral blood, represent a population of activated lymphocytes is not known. Analysis of DNA content showed that all sorted populations were in the resting phase of the cell cycle. Together with the inability to detect activation antigens in the killer population, these data provide evidence that cell activation is not requisite for competent killing function. In addition, difference in cell cycle, with respect to DNA content, does not account for nonkiller status in the free and binder fractions.

Poor recycling activity has been seen for NK cells in studies presented here and by others (12, 34). The level at which loss of function occurs has not been thoroughly investigated. Our results show that most killer cells retained some degree of granularity and indicate that loss of lytic machinery may not entirely explain poor recycling function. The refractory nature of killer cells can be explained, in part, by a partial loss of both binding and lytic potential.

Importantly, the NK-enriched preparation contained a minority of non-NK effector cells that could participate in conjugate formation and possibly lysis of K-562 cells. Accordingly, T cells were present in all the sorted populations ($\leq$10%); however, no significant enrichment was seen.

The ability to sort effector cells based on binding and lytic function and to examine the properties of these purified populations after activity represents an alternative strategy for understanding NK-cell biology. This technique can be applied to other effector-cell systems where cell interactions have direct and measurable consequences. Purification of functional subpopulations will allow direct examination of gene expression and response to various stimuli within each population. In addition, these purified populations should provide material for the identification of additional and specific surface proteins associated with each subpopulation.

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