

Natural Killer Lines and Clones with Apparent Antigen Specificity

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Summary

Fresh CD3⁻, CD16⁺ lymphocytes that adhered to selected allogeneic lymphoblastoid cell lines (LCL) were cultured with LCL in the presence of IL-2-containing medium. The resulting lines as well as clones derived from these lines expressed CD16 and/or CD56, but lacked detectable CD3 or TCR- α/β or TCR- γ/δ complexes on the cell surface. Northern blot analysis failed to detect CD3 ϵ or TCR- β transcripts, but revealed the presence of a TCR- γ chain transcript in one of these lines. In addition to displaying potent cytolytic activity against K562 erythroleukemia cells (a classical NK target), the vast majority of these lines and clones lysed their specific stimulator LCL to a significantly greater extent than irrelevant LCL. This selective killing was inhibited by the addition of cold stimulator LCL or K562 cells, or anti-LFA 1 mAbs, but not by irrelevant LCL or mAbs to CD3, class I or class II MHC antigens. These results indicate that some CD3⁻ lymphocytes, phenotypically indistinguishable from NK cells, can recognize and lyse allogeneic targets in a specific manner.

Natural killer (NK) cells are defined as lymphocytes that lyse certain transformed or virally infected targets without prior sensitization or restriction for products of the MHC (1, 2). Although T cells (CD3⁺) have been described with NK-like activity, classical NK cells are LGL that lack CD3 and express CD16 and/or CD56 (3, 4). Despite the fact that NK cells have a limited target cell range, no specific NK-associated surface receptor or target ligand has yet been defined, leading to speculation that such effector cells lack a highly refined antigen recognition system. However, recent studies from our laboratory suggest that some NK cells lyse their targets with a high degree of specificity (5). In those studies lymphocytes of NK phenotype (CD3⁻, CD16⁺) were cultured for several weeks with allogeneic microvascular endothelial cells (EC)¹ in the presence of IL-2-containing medium. The resulting cell lines, which retained their NK phenotype, lysed the original stimulating EC but not EC from unrelated donors. Similarly, Ciccone et al. recently described CD2⁺, CD3⁻ clones that lysed allogeneic lymphoid cells with apparent specificity, although the expression of CD16 and CD56 on these clones was not reported (6).

In the current study we attempted to define conditions for reproducibly generating allospecific effector lines from fresh cells of NK phenotype, that is, from classical NK cells. The

results suggest that at least some NK cells have the ability to interact with allogeneic targets in a cognate manner.

Materials and Methods

Monoclonal Antibodies and Immunofluorescence Analysis. mAbs used in this study were produced and purified in this laboratory and immunofluorescence staining was performed as described (7). Stained cells were analyzed in an Ortho Cytofluorograf System 50H cell sorter (Ortho Diagnostic Systems, Inc., Raritan, NJ).

Generation of NK Lines and Clones. PBL from healthy normal volunteers were isolated by Ficoll-Hypaque gradient centrifugation, and monocytes and B cells were removed by passage over nylon-wool columns. To obtain CD3⁻, CD5⁻ cells, cells devoid of monocytes and B cells were incubated with anti-CD3 mAb and applied to plastic petri dishes precoated with goat anti-mouse Ig (panning) as described (8). This procedure was repeated twice, and then the nonadherent cells were incubated with anti-CD5 mAb and panned on anti-mouse Ig to remove any residual CD5⁺ cells. The resultant lymphocyte population contained <0.5% CD3⁺ cells and >85% Leu 11c⁺ (CD16) cells by flow cytometric analysis.

To generate NK lines and clones, purified CD3⁻, CD16⁺ cells were incubated at 37°C for 1 h on a monolayer of irradiated (10,000 rad) LCL that had been bound to flat-bottomed microtiter wells with CELL-TAK (BioPolymers, Inc., Farmington, CT). Thereafter, cells not adherent to LCL were washed out and adherent cells were cultured in medium supplemented with IL-2-containing supernatant (conditioned medium) (9) at 37°C in 5% CO₂/air. After culture for 1 wk the growing cells were isolated on Ficoll-Hypaque gradients, and maintained as bulk cultures in 48-well plates,

¹ Abbreviations used in this paper: EC, endothelial cells; LCL, lymphoblastoid cell line.

or cloned at 0.7 cells/well in 96 microwells. Conditioned medium was added every 2-3 d, and 10^5 irradiated original LCL were also added weekly to each well. With this procedure multiple NK lines and clones were obtained and maintained for more than 6 mo.

Northern Blot Analysis. Total cellular RNA was prepared from fresh NK lines using the guanidinium thiocyanate/cesium chloride procedure. 15 μ g of total RNA was size fractionated in formaldehyde-1% agarose gels, transferred to nylon membranes, and hybridized with TCR or CD3 probes using methods described earlier (9). cDNA probes for TCR- β and - γ have been described (9). Two synthetic oligonucleotides were used to detect CD3 ϵ messages, whose sequences have been described (6).

Target Cells. The following cultured cell lines were used as targets in cytotoxicity assays: K562, a proerythroblastic cell line isolated from a patient with chronic myelogenous leukemia; and LCLs, ARENT (HLA-A2,2; B38,39; DR6,6), SKF (HLA-A24,30; B18,w35; DR5,5), MSAB (HLA-A1,2; B57,57; DR7,7), ND (HLA-A26,29; B7,55; DR9,10), LBF (HLA-A30,-; B13,-; DR7,-), and JEK (HLA-A2,11; B39,62; DR4,-). None of the LCLs had detectable surface Ig on the basis of immunofluorescent staining (data not shown).

Cytotoxicity Assays. NK cell lines and clones were tested for cytotoxicity in 4-h ^{51}Cr -release assays as described (9). In experiments designed to explore the role of cell surface molecules in cytotoxicity, mAbs were introduced and were present during the 4-h ^{51}Cr -release assays.

Cold Target Inhibition Assays. Target cells were labeled with ^{51}Cr as above and dispensed into 96-well plates at 2.5×10^3 cells/well. Unlabeled (cold) target cells were added to the wells at unlabeled/labeled target cell ratios ranging from 0:1 to 100:1. Effector cells were then added at various effector/labeled target cell ratios, and the plates were incubated at 37°C for a 4-h ^{51}Cr -release assay.

Results

CD3 $^-$,CD16 $^+$ cells were incubated for 1 h on LCL monolayers as described in Materials and Methods. Adherent cells were cultured with irradiated LCL in the presence of IL-2-containing medium, and following 6 wk of continuous expansion the resulting cell lines were analyzed in a flow cytometer for the expression of several surface markers. As shown in Fig. 1 for a representative line, all cells express CD2, CD16, and CD56, but lack expression of CD3, the TCR- α/β (antibody WT31) or TCR- γ/δ (antibody TCR- γ/δ -1). Multiple clones were derived by limit dilution culture of these lines, although the cloning efficiency was low (0.5-1.0%) and the growth rate slow (doubling time 48 h). Six clones were generated in sufficient numbers to study in parallel with parental cell lines. All were CD3 $^-$ and CD56 $^+$, but only three of six were CD16 $^+$ (data not shown). Transcription of TCR genes and the CD3 ϵ gene was studied by means of Northern blot analysis. As shown for two representative lines in Fig. 2, no message for TCR- β or CD3 ϵ was detectable. A TCR γ chain message was seen in one of three lines studied (two lines shown in Fig. 2 C). Identical results were obtained with a method for detecting specific transcripts based on the PCR (data not shown). In combination, the results of antibody staining and mRNA analysis rule out the possibility that conventional CD3 or TCR molecules are expressed by these cells.

To determine whether these cells had cytolytic activity, representative lines and clones were tested as effectors in 4-h ^{51}Cr -release assays against the original stimulator LCL as well as irrelevant LCLs and NK sensitive K562 cells. As shown in Fig. 3, all lines and clones exhibited potent, dose-dependent

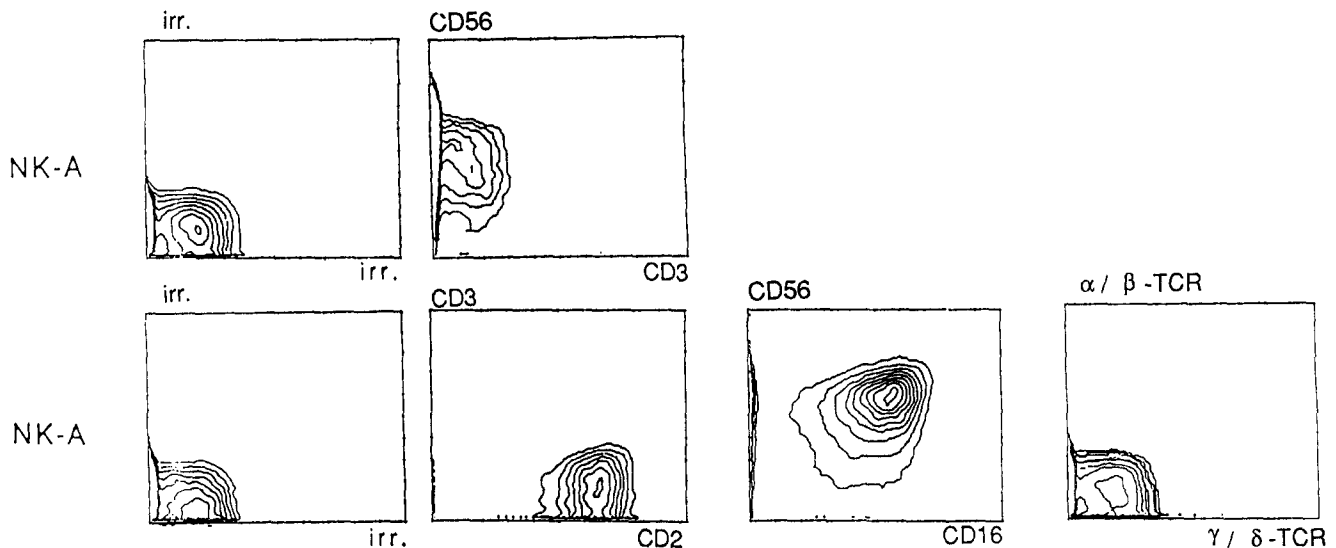


Figure 1. Two-color flow cytometric analysis of cell surface antigens on a cell line derived from fresh CD3 $^-$,CD16 $^+$ lymphocytes. After 6 wk of continuous expansion in the presence of irradiated LCL feeders, a representative cell line was separated from feeder cells over a Ficoll-Hypaque gradient and analyzed by two-color immunofluorescence in an Ortho System 50H cell sorter. The same staining pattern has been obtained repeatedly over a 6-mo period of continued propagation of this line.

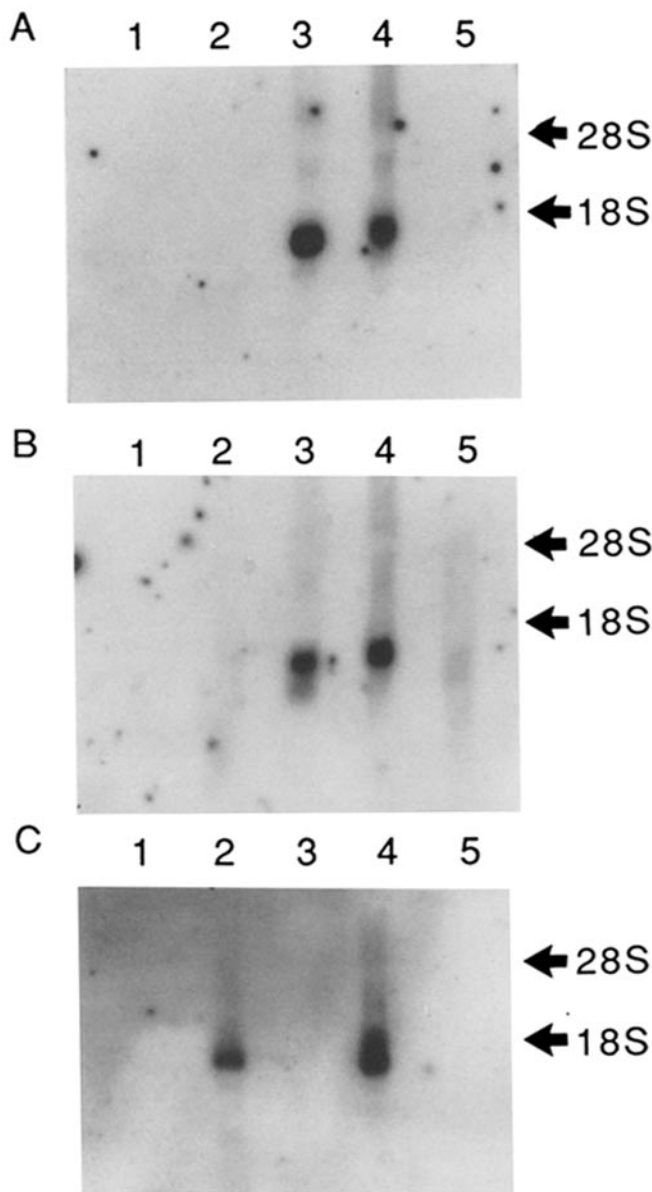


Figure 2. Northern blot analysis of TCR and CD3 ϵ gene products in antigen-specific NK lines. (A) CD3 ϵ , (B) TCR- β , (C) TCR- γ . (Lane 1) NK line 133; (lane 2) NK line 135; (lane 3) Jurkat leukemic cells; (lane 4) Peer leukemic cells; (lane 5) MSAB (B cell-LCL).

lysis of K562 cells. This is not surprising in light of the surface phenotype (CD3 $^-$,CD16 $^+$,CD56 $^+$, or CD3 $^-$,CD16 $^-$,CD56 $^+$) of the effectors. On the other hand, we were surprised to find that the lines and clones also lysed LCL targets (Fig. 3). Moreover, in the vast majority of experiments lysis of the original stimulator LCL exceeded that of the other LCLs. Similar results were obtained from NK lines or clones generated from multiple donors to three different stimulator LCL (Fig. 3), suggesting that the apparent specificity of lysis is not merely a consequence of differential sensitivity of the target LCL to NK or LAK cell-mediated lysis. Both

CD3 $^-$,CD16 $^+$ and CD3 $^-$,CD16 $^-$ lines demonstrated selective cytolysis of their stimulator LCL, making it unlikely that the CD16 molecule (Fc γ receptor) played a role in the lysis of these targets (data not shown).

To further analyze the specificity of cytolysis, the LCL-stimulated NK lines and clones were used as effectors in cold target inhibition assays. In these experiments, cold (unlabeled) target cells (K562, specific stimulator LCL, or irrelevant LCLs) were tested in varying numbers for the ability to inhibit cytolysis of ^{51}Cr -labeled NK-sensitive K562 cells or specific stimulator LCL. As shown in Fig. 4, A and C, specific killing of the original stimulator line, ARENT, was inhibited by the addition of unlabeled ARENT or K562 cells but not by the addition of irrelevant unlabeled LCL. As shown in Fig. 4, B and D, unlabeled K562 cells inhibited the lysis of ^{51}Cr -labeled K562 cells, whereas unlabeled ARENT cells had little if any inhibitory effect on the lysis of K562.

To explore the role of cell surface molecules involved in the NK killing (K562 target) and specific LCL killing by these lines and clones, cytotoxicity assays were carried out in the presence of selected mAbs. Only anti-CD11a (LFA-1 α), which is known to inhibit a variety of interactions between leukocyte effectors and their targets, completely inhibited killing of both targets (data not shown). An anti-CD18 (LFA-1 β) antibody partially inhibited lysis of both targets; however, inhibition was more potent when the specific LCL was tested. In contrast, antibodies to CD3, HLA class I, or HLA-DR at concentrations as high as 25 $\mu\text{g}/\text{ml}$ had no detectable effect on the lysis of either target.

Discussion

All of the CD3 $^-$,CD56 $^+$ and/or CD16 $^+$ lines and clones described in this report lysed the allogeneic LCL with which they had been cultured. Although absolute specificity for original stimulator LCL was not observed and all lines killed the classical NK-sensitive target, K562, in most instances the lines lysed their specific LCL stimulators to a greater extent than irrelevant LCL. The selective killing was not due simply to differences in sensitivity to cytolysis between various LCLs, since LCLs susceptible to lysis by some effector lines were not susceptible to lysis by others. Furthermore, the results cannot be explained by the presence of contaminating T cells in the cultures, since no CD3, TCR- α/β or TCR- γ/δ antigens could be detected by immunofluorescence analysis with a flow cytometer, and neither anti-CD3 nor anti-HLA mAbs, which are potent inhibitors of T cell-mediated lympholysis (9), inhibited the killing by these lines and clones.

Some NK cells and IL-2-dependent NK lines have been demonstrated to express truncated TCR- δ and - β transcripts (10, 11) as well as CD3 ϵ transcript (12). The CD2 $^+$,CD3 $^-$ allotypic lines described by Ciccone et al. had truncated TCR- β and CD3 ϵ messages (6). In contrast, our antigen-specific NK lines had no detectable CD3 ϵ or TCR- β messages, although one line expressed TCR- γ mRNA. While the explanation for these differences is unknown, the com-

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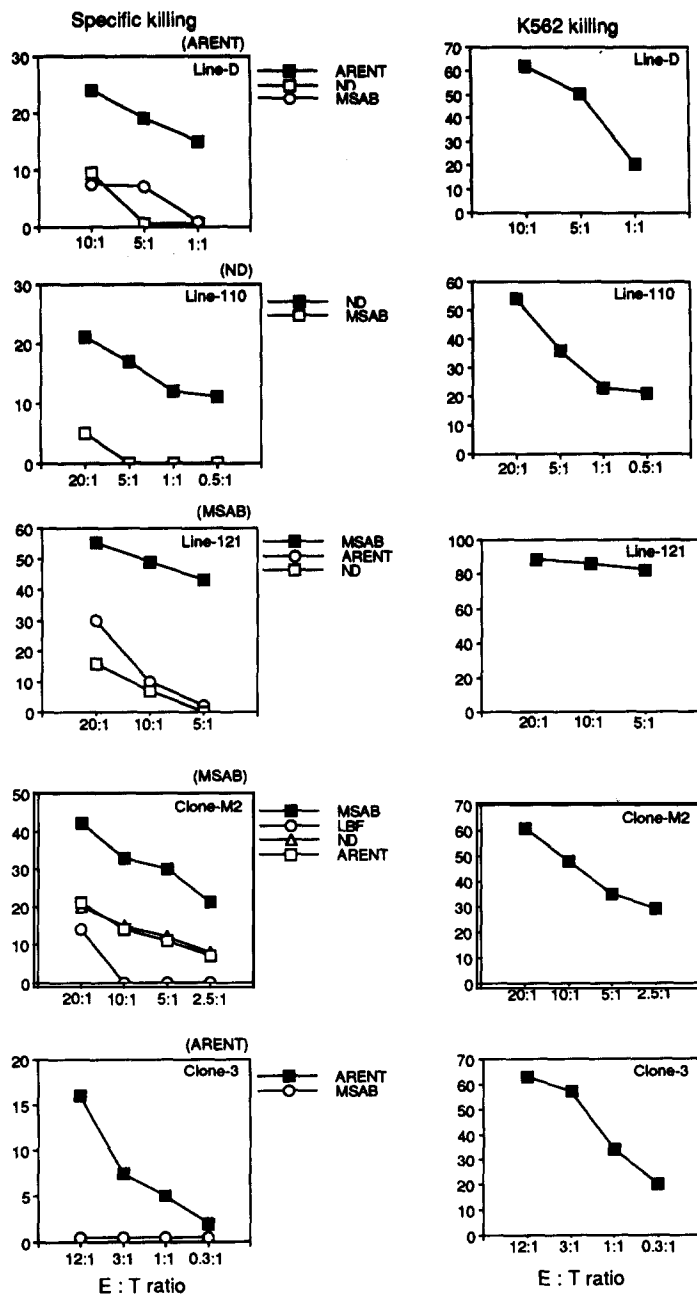


Figure 3. Cytotoxic activity of CD3⁻ cell lines and clones. Lines and clones generated as described in the text were tested for cytotoxic activity against the original stimulator LCL, irrelevant LCLs, and K562 cells, using a standard ⁵¹Cr-release assay as described in Materials and Methods. Values represent the mean percent killing of triplicate assays. The original stimulators for each line and clone are indicated in parentheses.

plete absence of TCR and CD3 transcripts from several of our lines suggests that the products of these genes are not required for either the allospecific or NK activity of the lines.

It is important to emphasize that the NK lines and clones described in this study were derived from CD3⁻, CD16⁺ cells that adhered to the particular LCL chosen for use as a stimulator. We have also generated NK lines and clones from single CD3⁻, CD16⁺ cells cultured without an initial adherence step in the presence of allogeneic LCL and high (>50 U/ml) concentrations of rIL-2. However, while this approach yielded a large number of rapidly growing NK clones, the vast majority of these clones failed to demonstrate specific lysis of their feeder LCL (data not shown). This suggests that

an initial adherence step as well as an avoidance of high concentrations of IL-2 in the culture medium may be important factors in the selective growth of the types of NK lines and clones described in this report.

Cold target inhibition studies confirmed the specificity of the NK lines and clones for their LCL stimulators. Thus, unlabeled (cold) stimulator LCL blocked the killing of the same targets which had been labeled with ⁵¹Cr, whereas LCL that were not lysed by the effector lines failed to inhibit the killing of specific targets. On the other hand, cold K562 cells not only inhibited the lysis of K562 but also inhibited the lysis of specific stimulator LCL, whereas none of the LCL blocked the killing of K562. One possible explanation for

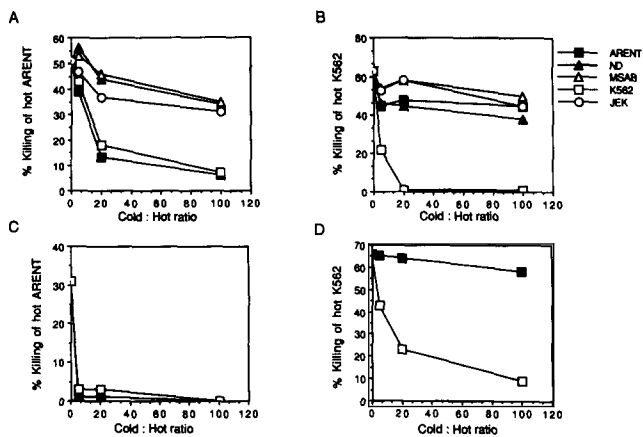


Figure 4. Effect of unlabeled (cold) target cells on the cytolytic activity of a CD3⁻ line and a CD3⁻ clone. Unlabeled target cells (K562, specific stimulator LCL ARENT, or irrelevant LCLs) were tested for their ability to inhibit the lysis of ⁵¹Cr-labeled specific stimulator LCL ARENT (panel A for the line-D and panel C for the clone-3) or K562 cells (panel B for the line and panel D for the clone). An effector to ⁵¹Cr-labeled target ratio of 12:1 for B, C, and D and 24:1 for A was maintained in all assays, as increasing numbers of cold inhibitor cells were added.

this pattern is that the avidity of the NK clones for K562 cells is substantially higher than their avidity for LCL targets, and binding of the clones to K562 cells prevents their access to LCL.

These results raise the interesting possibility that despite their lack of TCR- α/β or TCR- γ/δ structures, some NK cells interact with their targets in a cognate manner. Although there is little evidence in the literature to support this concept, it is noteworthy that previous attempts to absorb NK activity with target cells have suggested heterogeneity of both NK recognition and target structures (13, 14). Furthermore, NK cells have been shown to mediate hybrid resistance to parental bone marrow grafts (15, 16), a phenomenon that appears genetically restricted and directed at the products of the noncodominant hematopoietic histocompatibility (Hh) genes (17). These observations are consistent with the current results, as well as those of Ciccone et al. (6) and our previous findings in which endothelial cells were used as stimulators to generate target cell specific NK lines (5). Although the physiologic significance and molecular basis of these findings are unknown, our results suggest that the traditional view of NK cells as nonspecific effectors should be reconsidered.

We gratefully acknowledge the assistance of Claudia Benike, who provided helpful discussion and critical review of the paper, and Donna Jones, who provided superb secretarial support.

This work was supported in part by U.S. Public Health Service (National Institutes of Health) grants CA-24607 and AI-25922.

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Received for publication 5 September 1989 and in revised form 30 April 1990.

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