HUMAN NATURAL KILLER CELLS ISOLATED FROM PERIPHERAL BLOOD DO NOT REARRANGE T CELL ANTIGEN RECEPTOR β CHAIN GENES

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Natural killer cells are a population of lymphoid cells that are capable of killing certain tumor cell lines and virus-infected cells. NK cells are distinct from conventional CTL in that they are present in unimmunized hosts and their cytotoxic function does not require recognition of MHC antigens. In human peripheral blood, two essentially mutually exclusive cell populations can be identified using antibodies against the CD3 (Leu-4/T3) and CD16 (Leu-11) cell surface antigens (1). The CD3 antigen is a 22–29 kD glycoprotein complex associated with the T cell antigen receptor, an ~90 kD disulfide-linked hetero-dimer composed of an α and β subunit (2, 3). CD16, a 50–70 kD antigen associated with an Fc receptor for IgG, is present on essentially all peripheral blood NK cells (~10% of lymphocytes) and neutrophils, but is usually not expressed on B cells, T cells, eosinophils, or monocytes (1).

Recently, the genes coding for the mouse (3, 4) and human (5) T cell antigen receptor β chain have been cloned and sequenced. Like Ig genes in B cells, the T cell antigen receptor genes are composed of V, J, and C region elements that must be rearranged to produce a functional transcript (6). Two β genes, designated $\beta1$ and $\beta2$, are present in the genome of mouse and man (6, 7). $\beta1$ and $\beta2$ genes can both be productively rearranged and expressed. Rearrangement of β chain genes occurs early in ontogeny (8, 9), and most thymocytes, and all functionally competent mature T cells have been shown (3–11) to rearrange and express the C_β genes. In contrast, B lymphocytes and nonlymphoid cells usually do not rearrange C_β genes (3–11). Herein, we have isolated NK cells from human peripheral blood and examined the organization of the T cell antigen receptor C_β genes using restriction enzyme analysis.

Materials and Methods

Isolation of cells. Human peripheral blood from normal, healthy donors was obtained from the Stanford Blood Center, Stanford, CA. The mononuclear cell fraction was isolated using Ficoll/Hypaque. B cells and monocytes were depleted by adherence to plastic culture dishes and nylon wool (12). Nonadherent lymphocytes were centrifuged on a cushion of 40% Percoll, and the low density lymphocytes, enriched for NK cells, were harvested from the gradient interface (12). Low density lymphocytes were stained with FITC-conjugated anti-Leu-11a antibody, and separated into CD16⁻ (Leu-11⁻) and
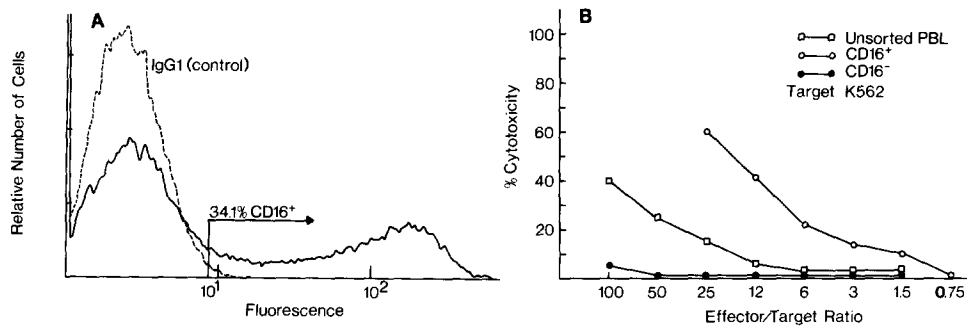
FIGURE 1. Isolation and NK cell–mediated cytotoxicity of CD16⁻ and CD16⁺ lymphocytes. NK cell–enriched PBL were stained with FITC-conjugated anti-Leu-11a antibody or FITC-IgG1 control antibody, and analyzed by flow cytometry. In A, the fluorescence histogram of FITC–anti-Leu-11–stained lymphocytes (solid line) is superimposed over the histogram of the FITC-IgG1 control sample (broken line). 34.1% of the low density lymphocytes expressed cell surface CD16 (Leu-11) antigen. Before enrichment on Percoll gradients, ~10% of the lymphocytes stained with anti-Leu-11 antibody. CD16⁻ (Leu-11⁻) and CD16⁺ (Leu-11⁺) lymphocytes were separated using a FACS (1). Reanalysis of the sorted populations indicated >98% purity. As shown in B, unsorted, CD16⁻ (Leu-11⁻) and CD16⁺ (Leu-11⁺) low density lymphocytes were tested for NK cell–mediated cytotoxicity against ⁵¹Cr-labelled K562 tumor cells in a 4-h radioisotope-release assay.

CD16⁺ (Leu-11⁺) fractions using a FACS 440 (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Granulocytes were obtained from the Ficoll/Hypaque pellet after hypotonic lysis of erythrocytes. Immunofluorescence staining, flow cytometry, data analysis, and cell sorting were performed as described previously (1).

Southern Blot Analysis. Genomic DNA were prepared from T cells, NK cells, granulocytes, and a human T leukemia cell line, HSB-2. 10 μg of each DNA were digested overnight with restriction enzymes (New England Biolabs, Boston, MA), electrophoresed in 0.8% agarose, and transferred to nitrocellulose membranes (Biorad Laboratories, Richmond, CA). The membranes were hybridized at 65°C for 24 h with the ³²P-labelled, nick-translated C_β probe (Oncor, Inc., Gaithersburg, MD) (~10⁸ cpm/μg) in hybridization buffer containing 6× SSC, 3× Denhardt's, 1 mM EDTA, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA (13). The membranes were washed three times (15 min, room temperature) in 10 mM sodium phosphate, 1 mM disodium EDTA, and 0.2% SDS, and were autoradiographed. Densiometric analysis was performed using a Quick Scan R&D (Helena Instruments, Beaumont, TX).

NK Cytotoxicity Assay. ⁵¹Cr-labeled K562 tumor cells were used as targets in a 4-h radioisotope-release assay (1).

Results and Discussion

Nonadherent low density human PBL, isolated as described in Materials and Methods, were stained with FITC anti-Leu-11a mAb and separated into CD16⁺ (Leu-11⁺) and CD16⁻ (Leu-11⁻) fractions using a FACS (Fig. 1A). Unsorted lymphocytes, CD16⁺ and CD16⁻ lymphocytes were tested for NK cell–mediated cytotoxicity against the NK-sensitive K562 cell line. As shown previously (1), essentially all cytotoxicity against the NK-sensitive tumor cell K562 was mediated by CD16⁺ cells (Fig. 1B). CD16⁻ lymphocytes were >99% T lymphocytes, based on staining with anti-Leu-4 (CD3) mAb, whereas <2% of the CD16⁺ lymphocytes coexpressed CD3.

Genomic DNA were prepared from granulocytes (germline control), HSB-2
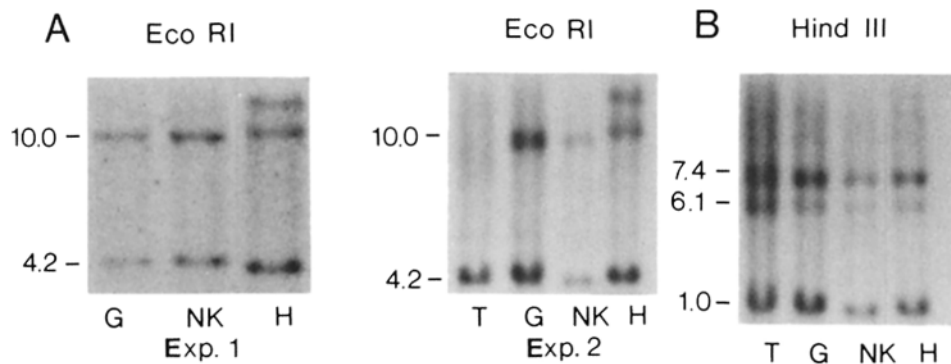
FIGURE 2.   Southern Blot analysis of CD16⁻ and CD16⁺ lymphocytes. Genomic DNA from CD16⁻ T cells (T), CD16⁺ NK cells (NK), granulocytes (G), or HSB-2 thymic leukemia cells (H) were digested with (A) Eco RI or (B) Hind III, and probed with a ³²P-labelled C_β cDNA. Data from two independent experiments using Eco RI are shown.

(a thymic leukemia cell line), peripheral blood T cells (CD16⁻), and peripheral blood NK cells (CD16⁺). DNA were digested with Eco RI, Hind III or Bgl II, transferred to membranes by Southern blot techniques, and probed with the ³²P-labeled C_β cDNA. In Eco RI–digested genomic DNA from granulocytes, the C_β probe hybridized with two distinct fragments of ~10 and 4 kb (Fig. 2A). Previous studies (7, 11, 14, 15) have established that the 10 kb and 4 kb fragments contain the C_β1 and C_β2 genes, respectively. HSB-2 thymic leukemia cells showed an Eco RI restriction enzyme pattern different than the germline pattern, indicating rearrangement of the C_β1 gene. The Eco RI restriction enzyme pattern of CD16⁺ NK cells was identical to the granulocytes. This was confirmed by densiometric analysis. The ratio of the density of the 10 kb to the 4 kb bands (1:1) from CD16⁺ NK cells was identical to that of granulocytes. These data demonstrate that the majority of peripheral blood NK cells do not rearrange the T cell antigen receptor C_β genes. In contrast, CD16⁻ peripheral blood T lymphocytes demonstrated essentially complete loss of the Eco RI C_β1 10 kb fragment. Loss of the 10 kb fragment containing the C_β1 gene is compatible with multiple individual rearrangements occurring in a polyclonal T cell population. Furthermore, these data suggest that both alleles of the C_β1 gene are rearranged or deleted in peripheral blood T lymphocytes (7, 14, 15). Lack of C_β rearrangement in the CD16⁺ population was confirmed in three independent experiments using NK and T cells isolated from different blood donors.

Both CD16⁺ NK cells and CD16⁻ T cells demonstrated a restriction enzyme pattern identical to granulocytes using Hind III (Fig. 2B). Similarly, NK cells, T cells, and granulocytes gave identical restriction enzyme patterns with Bgl II and Bam HI (not shown). The lack of detectable rearranged bands in a normal polyclonal T population using Hind III, Bam HI and Bgl II have been reported previously (14, 15).

These results are consistent with the recent findings of Flug et al. (14) who have proposed that loss of the Eco RI 10 kb C_β fragment in a mature, polyclonal population is a specific marker for T cell lineage. Since the genomic DNA

isolated from CD16$^+$ NK cells demonstrated a germline restriction enzyme pattern for the C$_\beta$ genes, indistinguishable from granulocytes, we conclude that at least the majority of NK cells do not express the T cell antigen receptor β chain. Further studies using probes for the V and J regions will be necessary to determine whether these elements are rearranged or productively expressed in NK cells. Due to the long distance (>10 kb) between the C and J regions of the T cell antigen receptor α gene, we have been unable to determine whether NK cells rearrange this gene using a C$_\alpha$ cDNA probe.

Reynolds et al. (16) have shown that rat leukemia cell lines with NK cell activity do not rearrange or transcribe C$_\beta$ genes. However, these findings contrast with reports that murine IL-2-dependent cell lines with NK activity do rearrange and express C$_\beta$ genes (17). Recently, Ritz and colleagues (18) have reported that some cloned human cell lines that mediated non–MHC restricted cytotoxicity rearrange and transcribe C$_\beta$ genes, whereas others do not. The non–MHC restricted cytotoxic cell lines that rearrange the C$_\beta$ genes express the CD3 antigen and a cell surface 90 kD T cell antigen receptor heterodimer (18). Antibodies against either CD3 or the clonally restricted heterodimer inhibit cytotoxicity (18). Cytotoxic cell lines that did not demonstrate C$_\beta$ rearrangement did not express the CD3 antigen.

Although cell lines and tumors are valuable models, it is important to establish whether or not they represent the normal, major cell population mediating a particular function. Results from this study clearly show that the major cell population mediating NK cell–mediated cytotoxicity, comprising ~10–15% of PBL, do not rearrange the C$_\beta$ T cell antigen receptor genes. The apparent discrepancies observed using cell lines can be explained by proposing that NK, i.e. non–MHC restricted cytotoxicity can actually be mediated by two distinct cell types. One is a unique subset of T cells that rearrange and express T cell antigen receptor genes and recognize target via a CD3/Ti complex. Although these non–MHC restricted cytotoxic T cells are rare in freshly isolated human peripheral blood, they can be expanded and selected for in IL-2-dependent cultures. The other type of non–MHC restricted cytotoxic cell is identified by expression of CD16 antigen and lack of CD3/Ti. These cells are relatively abundant in normal peripheral blood, and unlike T cells, do not rearrange T cell antigen receptor β genes or use this structure for target recognition. The unresolved question is how CD16$^+$ NK cells that lack T cell antigen receptor expression can recognize and lyse certain tumor cells and virus-infected cells.

The term NK cell has previously been used to describe these two distinct cell types that mediate non–MHC restricted cytotoxicity. This has created considerable confusion, since it implies that the two cell types either arise from the same lineage or represent maturational stages of a single cell type. There is no evidence to suggest a direct relationship between the CD3/Ti$^+$ and CD3/Ti$^-$ cells that mediate non–MHC restricted cytotoxicity. We suggest that it may be more appropriate to refer to the CD3/Ti$^+$ cells as non–MHC restricted CTL, since they are certainly within the T cell lineage and use the CD3/Ti complex to recognize antigen. NK cell should be used to describe CD3/Ti$^-$ (i.e. non-T) cytotoxic lymphocytes, since these lymphocytes are the major cell population mediating this activity.

Summary

The lineage of NK cells and their relationship to T lymphocytes have been controversial issues. Since rearrangement of the T cell antigen receptor β chain genes occurs early in the ontogeny and differentiation of all T cells, this can be used as an unequivocal marker to discriminate T from non-T lymphocytes. Recent studies (16–18) examining T cell antigen receptor gene rearrangement and expression in certain IL-2-dependent NK cell lines and leukemias have revealed that some lines rearrange $C\beta$ genes, whereas others do not. However, it is important to establish whether these cell lines are representative of the major population of NK cells freshly derived from the host. Herein, we have purified granulocytes, CD16$^+$ NK cells and T lymphocytes from human peripheral blood, prepared genomic DNA from each cell type, and then examined the organization of their T cell antigen receptor genes by restriction enzyme analysis using a $C\beta$ cDNA as probe. The $C\beta$ genes were in germline configuration in NK cells and granulocytes. In contrast, peripheral blood T lymphocytes showed rearrangement of the $C\beta$ gene. These data support the hypothesis that the majority of human peripheral blood NK cells are fundamentally distinct from T lymphocytes in lineage and nonself recognition.

References

1. Lanier, L. L., A. M. Le, J. H. Phillips, N. L. Warner, and G. F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J. Immunol.* 131:1789.
2. Meuer, S. C., K. A. Fitzgerald, R. E. Hussey, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function relationship to the T3 molecular complex. *J. Exp. Med.* 157:705.
3. Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis. 1984. Isolation of cDNA clones encoding T cell–specific membrane-associated proteins. *Nature (Lond.).* 308:149.
4. Hedrick, S. M., E. A. Nielsen, J. Kavaler, D. I. Cohen, and M. M. Davis. 1984. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature (Lond.).* 308:153.
5. Yanagi, Y., Y. Yoshikai, K. Leggett, S. P. Clark, I. Aleksander, and T. W. Mak. 1984. A human T cell–specific cDNA encodes a protein having extensive homology to immunoglobulin chains. *Nature (Lond.).* 308:145.
6. Gascoigne, N. R. J., Y. Chien, D. M. Becker, J. Kavaler, and M. M. Davis. 1984. Genomic organization and sequence of T-cell receptor β-chain constant- and joining-region genes. *Nature (Lond.).* 310:387.
7. Sim, J. E., A. Tunnacliffe, W. J. Smith, and T. H. Rabbitts. 1984. Complexity of human T-cell antigen receptor β-chain constant- and variable-region genes. *Nature (Lond.).* 321:541.
8. Snodgrass, H. R., P. Kisielow, M. Kiefer, M. Steinmetz, and H. von Boehmer. 1985. Ontogeny of the T-cell antigen receptor within the thymus. *Nature (Lond.).* 313:592.
9. Royer, H. D., O. Acuto, M. Fabbi, R. Tizard, K. Ramachandran, J. E. Smart, and E. L. Reinherz. 1984. Genes encoding the Ti β subunit of the antigen receptor undergo

rearrangement during intrathymic ontogeny prior to surface T3-Ti expression. *Cell.* 39:261.

10. Hedrick, S. M., R. N. Germain, M. J. Bevan, M. Dorf, I. Engel, P. Fink, N. Gascoigne, E. Heber-Katz, J. Kapp, Y. Kaufman, J. Kaye, F. Melchers, C. Pierce, R. H. Schwartz, C. Sorensen, M. Taniguchi, and M. M. Davis. 1985. Rearrangement and transcription of a T-cell receptor β-chain gene in different T-cell subsets. *Proc. Natl. Acad. Sci. USA.* 82:531.

11. Toyonaga, B., Y. Yanagi, N. Suciu-Foca, M. Minden, and T. W. Mak. 1984. Presence of T-cell receptor mRNA in functionally distinct T cells and elevation during intrathymic differentiation. *Nature (Lond.).* 311:385.

12. Phillips, J. H., N. L. Warner, and L. L. Lanier. 1984. Correlation of biophysical properties and cell surface antigenic profile of Percoll gradient–separated human natural killer cells. *Nat. Immun. Cell Growth Regul.* 3:73.

13. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 382–389.

14. Flug, F., P.-G. Pelicci, F. Bonetti, D. M. Knowles, and R. Dalla-Favera. 1985. T-cell receptor gene rearrangements as markers of lineage and clonality in T-cell neoplasms. *Proc. Natl. Acad. Sci. USA.* 82:3460.

15. Yanagi, Y., A. Chan, B. Chin, M. Minden, and T. W. Mak. 1985. Analysis of cDNA clones specific for human T cells and the α and β chains of the T-cell receptor heterodimer from a human T cell line. *Proc. Natl. Acad. Sci. USA.* 82:3430.

16. Reynolds, C. W., M. Bonyhadi, R. B. Herberman, H. A. Young, and S. M. Hedrick. 1985. Lack of gene rearrangement and mRNA expression of the β chain of the T cell receptor in spontaneous rat large granular lymphocyte leukemia lines. *J. Exp. Med.* 161:1249.

17. Yanagi, Y., N. Caccia, M. Kronenberg, B. Chin, J. Roder, D. Rohel, T. Kiyohara, R. Lauzon, B. Toyonaga, K. Rosenthal, G. Dennert, H. Acha-Orbea, H. Hengartner, L. Hood, and T. W. Mak. 1985. Gene rearrangement in cells with natural killer activity and expression of the β chain of the T-cell antigen receptor. *Nature (Lond.).* 314:631.

18. Ritz, J., T. J. Campen, R. E. Schmidt, H. D. Royer, T. Hercend, R. E. Hussey, and E. L. Reinherz. 1985. Analysis of T-cell receptor gene rearrangement and expression in human natural killer cell clones. *Science (Wash. DC).* 228:1540.