Brief Definitive Report

A SUBSET OF NATURAL KILLER CELLS IN PERIPHERAL BLOOD DISPLAYS A MATURE T CELL PHENOTYPE

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NK cells are defined by their ability to lyse target cells independent of restriction by major histocompatibility antigens and without prior immunization (1). Morphologically, NK cells appear to be a homogeneous population of large granular lymphocytes (LGL) (2), but phenotypic characterization of cell surface antigens has revealed considerable heterogeneity and has not clearly defined the relationship of these cells to other lymphoid and hematopoietic populations. The most specific NK-associated antigen appears to be NKH1, a 200-kD molecule, which is expressed on all NK cells in peripheral blood but only rarely expressed by other lymphoid or myeloid cells (3). Almost all NK cells express T11/E rosette receptor antigen (4); however, despite this finding, NK cells are considered to be a unique population that is distinct from CTL because the majority of NK cells in peripheral blood do not express T cell–like receptors for antigen (5). In contrast, studies with human NK clones (6, 7) have suggested that a subpopulation of T cells can mediate NK-like activity. In the present studies, we show that normal peripheral blood contains a small population of cells that express phenotypic, morphologic, and functional characteristics of both human NK cells and T cells.

Materials and Methods

Monoclonal Antibodies. Anti-T3, T4, T8, and T11 mAbs have been previously described (8). Anti-NKH1$_A$ is an IgM mAb with the same specificity as anti-NKH1 (N901) (3).

Analysis and Purification of Subpopulations of PBMC. Human PBMC were isolated from volunteer donors by Ficoll-Hypaque density gradient centrifugation. Two-color immunofluorescence analysis was accomplished by incubating cells for 30 min at 4°C with fluorescein-conjugated anti-NKH1$_A$ and a phycoerythrin-conjugated mAb (either anti-T3, T4, or T11 provided by Coulter Immunology, Hialeah, FL). In some experiments, anti-NKH1$_A$-FITC was used simultaneously with anti-T8 biotin (Coulter Immunology) followed by the addition of avidin conjugated phycoerythrin (AV-PE; Becton Dickinson & Co., Mountain View, CA). Background fluorescence was determined by incubating cells with a nonreactive fluorescein-conjugated antibody and either a nonreactive phycoerythrin-conjugated antibody or a nonbiotinylated antibody followed by AV-PE.

The simultaneous analysis of green and red fluorescence was obtained from a single

Reinhold E. Schmidt is a recipient of a fellowship (Schm 596/1-1) from the Deutsche Forschungsgemeinschaft. Jerome Ritz is a Scholar of the Leukemia Society of America. This work was supported in part by National Institutes of Health grant CA-41619.

TABLE I

Expression of T Cell Antigens on $NKH1_A^+$ Cells in Normal Peripheral Blood

Donor	$NKH1_A^+$	$T3^+$ $NKH1_A^+$*		$T4^+$ $NKH1_A^+$		$T8^+$ $NKH1_A^+$		$T11^+$ $NKH1_A^+$	
		Total	$NKH1^+$	Total	$NKH1^+$	Total	$NKH1^+$	Total	$NKH1^+$
	%	%	%	%	%	%	%	%	%
1	19.4	1.9	9.8	0.4	2.3	—	—	11.3	53.0
2	12.4	4.7	38.1	0.8	8.7	2.7	24.4	13.0	87.0
3	8.3	2.7	31.8	0.2	2.9	—	—	9.5	100.0
4	11.4	2.4	21.0	0.6	5.5	—	—	7.0	68.3
5	7.7	1.2	15.2	1.0	14.8	2.1	25.4	10.0	67.2
6	8.2	2.3	28.1	0.5	4.8	2.9	29.1	6.8	78.1
Mean	11.2	2.5	24.0	0.6	6.5	2.6	26.3	9.6	75.6

* Number of cells expressing both cell surface antigens was determined by direct immunofluorescence and simultaneous two-color analysis using an EPICS V. 10,000 cells were analyzed in each experiment and results are expressed as percent of total PBMC and as a percent of the number of $NKH1^+$ cells.

laser exciting both FITC and PE at 488 nm using an Epics V (Coulter Electronics, Hialeah, FL). Electronic compensation for the small overlaps of green and red fluorescence yield signals essentially identical to those with either reagent alone. 10,000 cells were analyzed in each sample and results displayed as an orthographic projection plotting log green versus log red fluorescence. In the sorting experiments, $NKH1^+T3^+$ doubly fluorescent cells were separated from $NKH1^+T3^-$ (green only) and from $NKH1^-T3^+$ (red only) cells. The purified cells were sorted in RPMI 1640 plus 10% pooled human AB serum at a rate of 3,000 cells/s. Cells were then washed twice and resuspended in RPMI 1640 containing 5% AB serum for functional assays and cytocentrifuge smears.

Cytotoxicity Assays. Cytotoxicity assays were performed using a standard chromium release method previously described (3). In the blocking studies with anti-T3, purified effector cells were cultured overnight at 37°C after cell sorting in the presence of anti-T3 or control antibody (anti-$NKH1_A$). Subsequently, mAbs were also added to individual wells for the 4 h cytotoxicity assay.

Culture of Cell Sorter–purified Subpopulations. $NKH1_A^+T3^+$ cells purified by cell sorting were cultured on a feeder layer of irradiated (5,000 rad) allogeneic PBMC plus EBV-transformed B cells and 10% lymphocyte-conditioned medium. After 10–14 d, the polyclonal cell lines contained 60–80% $T3^+NKH1^+$ cells. These cells were then resorted as described above, to obtain a population that was >95% $T3^+NKH1^+$. Resorted cells were then used for cytotoxicity assays without further in vitro culture.

Results

Two-Color Analysis of Peripheral Blood Lymphocytes. In order to evaluate the coexpression of NKH1 and T cell antigens, PBMC from six different individuals were examined (Table I). The mean percent of $NKH1^+T3^+$ cells in peripheral blood was 2.5% of PBMC. This represents ~24% of the $NKH1^+$ population. The T4 antigen is very rarely coexpressed with NKH1 and <1% of total PBMC express this phenotype. $NKH1^+T8^+$ cells compose ~2.6% of total PBMC and this represents 26.3% of all $NKH1^+$ lymphocytes, a number quite similar to the percentage of $NKH1^+T3^+$ cells. It is thus likely that many $NKH1^+T3^+$ lymphocytes also coexpress T8 antigen. In this regard, it should be noted that almost all NK-active clones that express both NKH1 and T3 also coexpress T8 antigen. $NKH1^+T11^+$ lymphocytes make up 9.6% of total PBMC. Unlike other T cell–associated antigens, $T11^+$ cells represent the great majority of the $NKH1^+$ subset.

Purification of $NKH1^+T3^+$ Cells. After showing the presence of $NKH1^+T3^+$ lymphocytes in peripheral blood, we used immunofluorescent cell sorting to

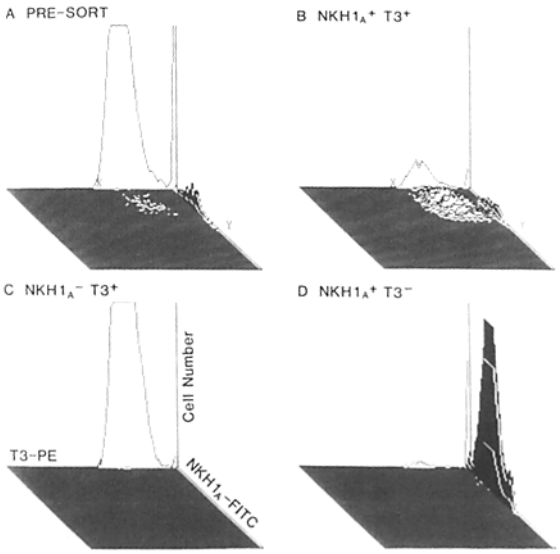

FIGURE 1. Two-color immunofluorescence cell sorter purification of peripheral blood lymphocyte subpopulations. (*A*) Pre-sort analysis with anti-NKH1$_A$-FITC (*y*-axis) and anti-T3-phycoerythyrin (*x*-axis); (*B*) post-sort analysis of purified NKH1$^+$T3$^+$ cells; (*C*) post-sort analysis of purified NKH1$^-$T3$^+$ cells; (*D*) post-sort analysis of purified NKH1$^+$T3$^-$ cells.

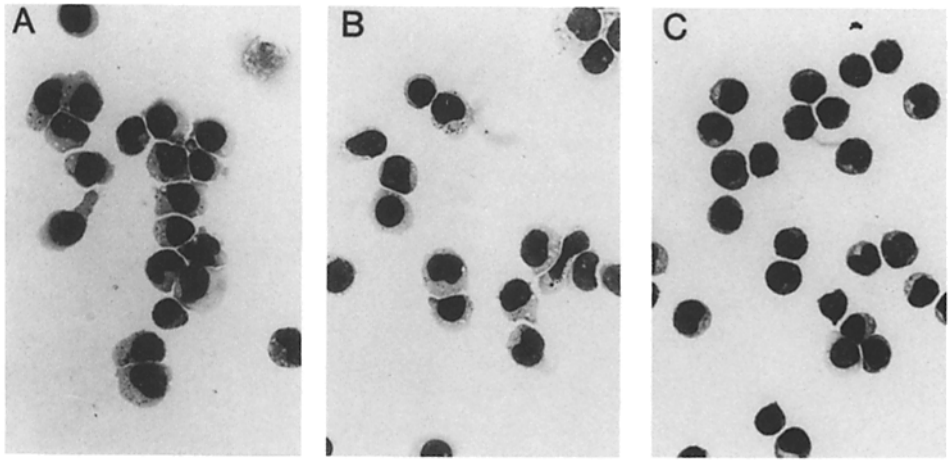

FIGURE 2. Morphology of NKH1$^+$T3$^-$ (*A*), NKH1$^+$T3$^+$ (*B*), and NKH1$^-$T3$^+$ (*C*) lymphocyte populations. Cytocentrifuge smears of cell sorter–purified populations were stained with Wrights Giemsa.

purify these cells for further characterization. Fig. 1*A* shows the presence of a small population of NHK1$^+$T3$^+$ cells in a representative sample before cell sorting. Fig. 1*B* shows the post-sort analysis of the purified NKH1$^+$T3$^+$ cells. In this experiment, 84% of sorted cells coexpress T3 and NKH1. The post-sort analysis of NKH1$^-$T3$^+$ and NKH1$^+$T3$^-$-purified cells is shown in *C* and *D*. As shown in Fig. 2, both NKH1$^+$T3$^-$ cells (Fig. 2*A*) and NKH1$^+$T3$^+$ cells (*B*) have

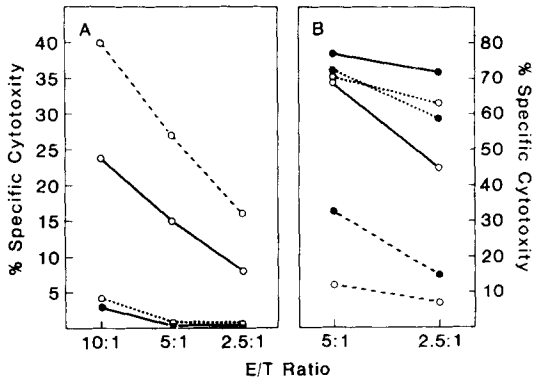

FIGURE 3. NK activity of NKH1$^+$T3$^+$ lymphocytes and inhibition by anti-T3 antibody. (*A*) Cytolytic activity of purified subpopulations obtained from peripheral blood. Specific cytotoxicity was measured at various E/T cell ratios for NKH1$^+$T3$^+$ (—), NKH1$^+$T3$^-$ (---), and NKH1$^-$T3$^+$ (....) cells. (○) cytotoxic activity in the presence of media only. (●) cytotoxicity after addition of anti-T3 antibody. (*B*) Cytolytic activity of cultured NKH1$^+$T3$^+$ lymphocytes. Cytotoxicity assays were performed in the presence of media (—), anti-NKH1$_A$ (....), or anti-T3 (---) mAb. Results were obtained from two different experiments: sort *A* (○) and sort B (●). K562 target cells were used in all experiments.

the typical structural appearance of LGL, whereas NKH1$^-$T3$^+$ cells (*C*) show the characteristics of small T lymphocytes.

NK Activity of NKH1$^+$T3$^+$ Lymphocytes. When tested against K562 target cells, purified NKH1$^+$T3$^+$ cells mediate cytotoxicity at relatively low E/T ratios (Fig. 3*A*). For comparison, there was some what higher activity in the NKH1$^+$T3$^-$ population, but no cytotoxicity was mediated by NKH1$^-$T3$^+$ cells. Similar results were seen in four of five different experiments, but in one experiment, both NKH1$^+$T3$^+$ and NKH1$^+$T3$^-$ populations exhibited equivalent levels of cytotoxicity. Also shown in Fig. 3 A, addition of monoclonal anti-T3 can completely abrogate the NK activity of NKH1$^+$T3$^+$ cells. In control experiments, anti-T3 did not abrogate NK activity on NKH1$^+$T3$^-$ cells and anti-NKH1$_A$ did not inhibit cytotoxicity of either NKH1$^+$T3$^+$ or NKH1$^+$T3$^-$ cells (data not shown).

The effect of anti-T3 was also evaluated with NKH1$^+$T3$^+$ lymphocytes that had been cultured in vitro for 14 d. After culture, cells were re-sorted to ensure the purity of the effector cell population before assessment of cytotoxicity. As shown in Fig. 3*B*, cultured NKH1$^+$T3$^+$ cells display a high degree of cytotoxicity against K562 targets. Addition of anti-NKH1$_A$ does not inhibit cytolytic activity, but incubation with anti-T3 results in marked inhibition. Addition of either anti-T8 or anti-Ia mAbs has no effect on cytotoxicity of NKH1$^+$T3$^+$ cells (data not shown).

Discussion

After purification of NKH1$^+$T3$^+$ cells, we showed that this subset displays spontaneous cytotoxicity as well as the characteristic LGL morphology of NK-active cells. Comparing NKH1$^+$T3$^-$ and NKH1$^+$T3$^+$ cells, both exhibit typical LGL morphology, but in some individuals, azurophilic granules appear more pronounced in the NKH1$^+$T3$^-$ subset. This finding is consistent with results of

functional assays, since $NKH1^+T3^-$ cells display somewhat higher spontaneous cytotoxicity than $NKH1^+T3^+$ cells. Nevertheless, both $NKH1^+$ fractions maintain high levels of NK activity compared with $NKH1^-T3^+$ cells, which do not kill K562 targets.

Since T3 antigen is noncovalently associated with the T cell receptor for antigen (Ti), and is required for surface membrane expression of Ti-like structures, we used anti-T3 antibody to determine whether $NKH1^+T3^+$ NK cells were functionally related to T cells. As described previously for $T3^+$ NK clones (6), cytotoxicity of $NKH1^+T3^+$ NK cells could be blocked by anti-T3. Since incubation with anti-T3 results in comodulation of both T3 and Ti these results suggest that $T3^+NKH1^+$ cells use a T cell receptor–like structure to interact with specific targets. These results were confirmed when purified $NKH1^+T3^+$ cells were cultured for 14 d, re-sorted, and then tested for NK activity. Cultured cells maintained a high degree of cytolytic activity, which could be inhibited by anti-T3 but not by antibodies specific for NKH1, T8, or Ia antigens.

Although the majority of NK cells do not express T3, there have been previous indications that a subset of NK cells is functionally related to T lymphocytes. In particular, recent studies of both human and murine NK clones have shown that some clones have a mature T cell phenotype, express clonotypic receptors for antigen, and have functional rearrangement and expression of T cell receptor genes (6, 9, 10). Although the analysis of cultured $T3^+$ NK clones may reflect some degree of in vitro selection of cells with a complete T cell phenotype, we have recently shown that clonotypic antigens of NK clones can also be found in low frequency in uncultured peripheral blood (11). Lymphokine activated killer (LAK) cells, may also represent an expansion of this population of cells. LAK cells have been shown to express a T cell phenotype after in vitro culture (12), and the majority also express NKH1.

Although some NK clones appear to have functional characteristics of mature T cells, the majority of NK clones do not express T3 antigen or T cell receptors for antigen, and do not have functional rearrangements of T cell receptor genes (9). Similarly the majority of NK cells in peripheral blood do not express T3 and do not appear to use T cell–like receptors for target antigen (5). $NKH1^+T3^+$ NK cells therefore appear to be a unique and functionally distinct population of NK cells, which can be distinguished from cytotoxic T cells as they can kill a variety of targets in an MHC-independent fashion. However, despite their broad reactivity, the present studies also suggest that this subset of NK cells can mediate their functional activity through a T cell receptor–like structure.

Summary

Normal human PBMC were analyzed for the presence of cells expressing both T3 and NKH1 antigens, using direct two-color immunofluorescence. In six individuals, $NKH1^+T3^+$ cells were found to represent 2.5% of PBMC and 24% of the total number of $NKH1^+$ cells. Purified $NKH1^+T3^+$ cells were shown to have the typical morphology of large granular lymphocytes (LGL). $NKH1^+T3^+$ cells also exhibited spontaneous cytotoxicity against K562 target cells and this lytic activity could be inhibited by anti-T3 mAb. Similar results were obtained with $NKH1^+T3^+$ cells cultured in vitro in lymphocyte-conditioned medium.

Taken together, these results indicate that $NKH1^+T3^+$ cells represent a unique population of NK-active cells in normal peripheral blood. Although these cells exhibit LGL morphology and NK activity, this appears to be mediated through a functional T cell–like receptor for target antigen.

We thank John Caulfield, Ph.D., for providing photographs of the purified cell fractions and Elizabeth H. Swinton for secretarial assistance.

Received for publication 10 March 1986.

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