EXPRESSION OF THE NKTa CLONOTYPE IN A SERIES OF HUMAN NATURAL KILLER CLONES WITH IDENTICAL CYTOTOXIC SPECIFICITY

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NK cells, which appear morphologically as a homogeneous population of large granular lymphocytes (LGL),[1] have been implicated in a number of diverse immunologic functions including cytotoxicity against tumor cells and virally transformed cells, regulation of immunoglobulin production, and regulation of hematopoiesis (1–4). However, despite their homogeneous morphology, NK cells comprise a small heterogeneous population of peripheral blood lymphocytes (5, 6). In part, the heterogeneity of NK cells has become apparent through characterization of the cell surface antigens expressed on these cells using various mAbs (7–9). These studies have shown that almost all NK cells express NK-associated antigens such as NKH1 and B73.1, but that other antigens, such as HNK-1 and NKH2, are only expressed to varying degrees on subsets of LGL (10, 11). The vast majority of NK cells express the T11/E rosette receptor antigen, but T8, another T cell associated antigen, is only expressed on ~30% of NK cells (12). Although numerous studies have indicated that mature T cells are capable of NK-like activity, very few peripheral blood NK cells have been found to express T3 antigen, T cell receptor (Ti) like structures, or mature T cell antigens such as T1 and T12 (7).

In recent studies, we have developed a series of cloned human NK cell lines. Phenotypic analysis of these cells has revealed that, like uncultured NK cells, the majority of NK clones express the T11 E rosette antigen and the pan-NK cell antigen NKH1, but do not express T3 antigen or functional T cell receptor-like structures (8, 13). However, unlike the majority of NK cells in peripheral blood, a number of NK clones were found to express a mature T cell phenotype including T3 and T8 antigens in addition to T11 and NKH1. Further charac-

This work was supported in part by National Institutes of Health grant CA 34183. Dr. Schmidt is a recipient of a fellowship from the Deutsche Forschungsgemeinschaft (Schm 596/1-1). Dr. Ritz is a Scholar of the Leukemia Society of America. Dr Schmidt's present address is Abt. Immunologie und Transfusionsmedizin, Zentrum Innere Medizin und Dermatologie, Med. Hochschule Hannover, Postfach 610180, D-3000 Hannover 1, West Germany.

[1] *Abbreviations used in this paper:* F/H, Ficoll Hypaque; LCM, lymphocyte conditioned media; PBMC, peripheral blood mononuclear cells; LGL, large granular lymphocyte; SSPE, standard phosphate saline EDTA; *Ti*, T cell receptor.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/86/04/0812/14 $1.00
Volume 163 April 1986 812–825

terization of these clones showed that three T cell-like NK clones, JT9, JT10, and JT11, derived from the same individual expressed the same clonotypic Ti-like structure, termed NKTa (14, 15). The subsequent identification of a 140-kD cell surface activation antigen termed TNK_{TAR} as the target antigen for NK clones expressing the NKTa clonotype, confirmed the MHC-independent cytolytic specificity of these cells but did not establish the functional relevance of this population as a mediator of NK activity in vivo (16). In order to determine whether the $T3^+NKTa^+$ lymphocyte population is an NK active subset present in unstimulated peripheral blood, we examined fresh PBMC from the donor of clones JT9, JT10, and JT11 for the presence of cells expressing the NKTa clonotype. After finding a small but distinct population of cells reactive with anti-NKTa, we used flow cytometric cell sorting and in vitro cloning procedures to establish additional clones that maintain NK-like activity and express the NKTa clonotype. This report describes the detailed, phenotypic, functional, and genetic analysis of this new series of NK clones.

Materials and Methods

Monoclonal Antibodies. mAbs used in these studies have been previously described in detail. T3 and T12 are pan-T cell antigens expressed on all mature T lymphocytes in human peripheral blood (17). T4 is expressed on a subset of peripheral T cells having primarily inducer/helper function. T8 antigen is expressed on a subset of peripheral T cells having primarily cytotoxic/suppressor function. Anti-T11 defines an antigen associated with the sheep erythrocyte receptor (18). Mo1 antigen has been shown to be the cell surface receptor for C3bi (19). NKH1 has been defined as a pan-NK cell antigen, whereas NKH2 has been shown to identify a subset of LGL with relatively low NK activity in unstimulated peripheral blood (11). B73.1 defines the FcR2 receptor present on NK cells and granulocytes (kindly provided by Dr. B. Perussia, Wistar Institute, Philadelphia, PA) (10). HNK1 antigen is expressed on ~60% of NK cells in normal peripheral blood and on some T lymphocytes that do not have NK activity (11, 20). Anti-transferrin receptor antibody (5E9) was obtained from American Type Culture Collection, Rockville, MD. The mAbs anti-NKTa and anti-NKTb have been shown to be specific for a T3-associated clonotypic T cell receptor-like structure on cloned human NK cell lines (14, 15). The mAbs anti-TNK_{TAR} (16) and 4F2 (21) define a 140-kD early activation antigen on hematopoietic cells.

Phenotypic Analysis of Cell Surface Antigens. Human peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteer donors by Ficoll-Hypaque (F/H) density gradient centrifugation.

Phenotypic analysis of normal PBMC and cloned cell lines was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse Fab IgG as described previously (11). Samples were analyzed on an Epics V or an Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL). 10,000 cells were analyzed in each sample and the results presented as histograms which display the number of cells (ordinate) versus the intensity of fluorescence (abscissa) expressed on a logarithmic scale for each reagent. The negative control used to determine background fluorescence was an ascites derived from a nonreactive hybridoma. mAbs were always used at saturating concentrations (1:100–1:500). In experiments that evaluated the presence of $NKTa^+$ cells in normal PBMC, 100,000 cells were analyzed with anti-NKTa and with negative ascites.

Purification of $NKTa^+$ Cells. Before flow cytometric sorting of $NKTa^+$ cells, an immune rosetting technique was used to enrich for this population of cells. Nonadherent PBMC were incubated at a concentration of 25×10^6 cells/ml for 30 min at 4°C with saturating amounts of anti-NKTa. Unbound antibody was removed by two wash steps and mAb-treated cells were mixed with a 10% (vol/vol) solution of anti-Ig coated ox erythrocyte suspension at a ratio of 0.75 ml for 10^8 PBL to form immune rosettes. The mixture was

pelleted (300 *g*, 10 min) and incubated for 20 min at 4°C. The ox red cell PBMC mixture was resuspended vigorously and rosetted cells were separated from nonrosetting cells by F/H density gradient sedimentation. Rosetted $NKTa^+$ cells were recovered from the F/H pellet by hypotonic lysis. Subsequently, cells were washed twice and the immune rosetting procedure was repeated with the NKTa-enriched fraction. The entire procedure was performed in RPMI 1640 containing 5% pooled human AB serum at 4°C. Viability was consistently >95% in all cell fractions. After an overnight incubation at 4°C, rosette positive cells were separated into $NKTa^+$ and $NKTa^-$ fractions using a cell sorting procedure. Briefly, rosette positive cells were incubated with anti-NKTa for 30 min at 4°C, washed twice, and then incubated with GM-FITC for an additional 30 min at 4°C. After two additional washes, $NKTa^+$ fluorescent cells were separated from nonfluorescent cells using an Epics V (Coulter Electronics Inc.). Background fluorescence was determined with a nonreactive hybridoma ascites, and positive cells were sorted in 10% pooled human AB serum at a rate of 3,000 cells/s. Cell sorter purified $NKTa^+$ cells were either cloned directly into 96 well plates at 1 cell/well using the Epics V autocloning procedure, or by limiting dilution technique plating 1 cell/well. In either case, cells were cloned into sterile V bottom microtiter plates.

Generation of Human Cloned Cell Lines. Methods for generation of human NK cloned cell lines have been described in detail (22). Briefly, $NKTa^+$ cells obtained by immunofluorescent sorting were cloned at 1 cell/well on a feeder layer of allogeneic irradiated (5,000 rad) PBMC plus allogeneic irradiated (5,000 rad) EBV transformed B cells. All colonies were expanded by addition of fresh culture medium every 3 d. Culture medium was RPMI 1640 supplemented with 1% penicillin-streptomycin, 1% sodium pyruvate, 20% human AB serum, and 10% lymphocyte conditioned medium (LCM). Preparation of LCM containing IL-2 has been described previously (11). All cell lines used in these studies have been subcloned at least three times at 100 cells/well on feeder layers of allogeneic irradiated PBMC plus EBV transformed B cells.

Clones CNK11, CNK12, CNK13, CNK14, and CNK15 were derived from cloning of purified $NKTa^+$ cells from normal peripheral blood. Clones CNK8 and CNK9 were derived from the same individual in experiments performed 6 mo before the sorting of $NKTa^+$ cells. These two clones were generated following the cell sorter purification and cloning of $NKH2^+$ cells from PBMC (11, 13). NK clones JT9, JT10, and JT11 have been described in detail (14–16). Each of these clones was derived at different times from the same individual and have previously been shown to express the NKTa clonotype. CNK3 is a $T3^+$, $T11^+$, $NKH1^+$, $NKH2^+$, $NKTa^-$ NK clone from a different donor (23). JT3 is a $T3^-$, $T11^+$, $NKH1^+$, $NKH2^+$, and JT_B18 is a $T3^-$, $T11^+$, $NKH1^+$, $NKH2^-$ NK clone. Both clones have been described in detail previously (8, 11). CNK6 is a $T3^-$, $T11^+$, $NKH1^+$, $NKH2^+$ clone, which was also generated from $NKH2^+$ sorted cells.

Cytotoxicity Assays. Cytotoxicity assays were performed according to a standard chromium release method previously described (23). All experiments were done in triplicate using V bottom microtiter plates. Medium was RPMI 1640 plus 5% pooled human AB serum and 1% penicillin-streptomycin. Assays were performed at various E/T ratios using 5,000 target cells/well. REX and Molt-4 are T cell leukemia cell lines, whereas K562 is a myeloid cell line established from a patient with chronic myelogenous leukemia. Daudi is a Burkitt lymphoma cell line. U937 cells were derived from a monocytic leukemia and HL-60 cells from a promyelocytic leukemia. Laz 461 is an EB virus transformed B cell line derived from the same donor as the $NKTa^+$ NK clones.

Inhibition studies were performed at an E/T ratio of 5:1 and by preincubating either the effector cells with anti–NKTa, anti–NKTb, anti–T3, or anti–NKH1 mAb for 30 min at 37°C, or by preincubation of the target cells with anti-TNK_{TAR}, 4F2, both mAbs, or 5E9 antibody for 30 min at room temperature.

Southern Blot Analysis. DNA from the NK clones and an EBV transformed B cell line (Laz 461) was extracted using standard methods (24, 25). DNA was digested with either Eco RI or Bam HI, fractionated on 0.8% agarose gels, transferred to nitrocellulose membrane, and hybridized with ^{32}P-labeled *Ti* $C\beta$ probe. The probe specific for the constant region gene of *Ti* β REX ($C\beta$ REX) used in southern blot analysis was purified

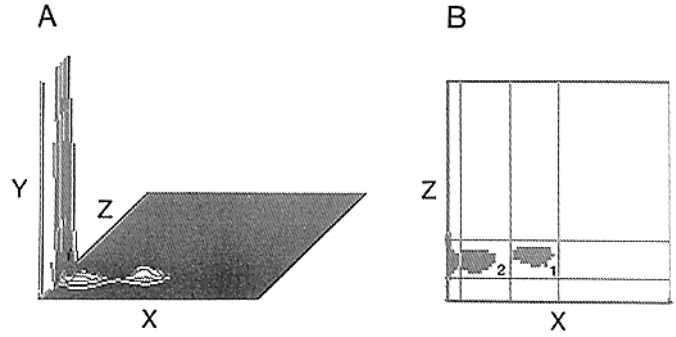

FIGURE 1. Identification of peripheral blood lymphocytes reactive with anti-NKTa mAb. Nonadherent PBMC were enriched for NKTa$^+$ cells by immune rosetting with anti-NKTa-coated ox RBC. Enriched cells were subsequently incubated with additional anti-NKTa antibody, stained with goat anti-mouse Ig FITC and 100,000 cells analyzed using an Epics V flow cytometer. (*A*) In this three-dimensional analysis, the *X*-axis represents fluorescence intensity on a log scale, the vertical *Y*-axis represents cell number, and the *Z*-axis represents cell size. (*B*) The dot matrix analysis for the same population is also shown. Here the *X*-axis represents fluorescence intensity on a log scale and the *Z*-axis represents cell size. Region *1* represents a discrete population with high intensity expression of NKTa antigen, whereas region *2* represents a relatively low degree of binding of anti-NKTa which is likely to be nonspecific.

from pβREX with Eco RI and Bgl II (New England Biolabs, Beverly, MA) and labeled with ^{32}P by nick translation. The isolation of this cDNA clone encoding Ti Cβ REX has been described in detail previously (26). The filters were hybridized in 6× standard phosphate saline EDTA (SSPE) at 68°C overnight, washed in 1× SSPE plus 0.1% SDS at room temperature for 30 min, 0.5× SSPE plus 0.1% SDS at 68°C for 30 min, and then twice in 0.2× SSPE plus 0.1% SDS at 68°C for 60 min, and membranes were exposed to Kodak XR film (Eastman Kodak Co., Rochester, NY) overnight.

Results

Identification of NKTa$^+$ Cells in Unstimulated Peripheral Blood. In earlier studies it was found that three NK clones generated at three different times from the same individual during a period of two years revealed an identical mature T cell-like phenotype (T3$^+$T8$^+$T11$^+$NKH1$^+$NKTa$^+$), and identical cytotoxic specificity (14, 15). In the present studies, we examined the peripheral blood of the same individual for the presence of cells expressing the NKTa clonotype. Using indirect immunofluorescence reactivity with monoclonal anti-NKTa antibody, flow cytometric analysis of 100,000 PBMC indicated the presence of only 323 NKTa$^+$ cells. Since control cells incubated with negative ascites and GM-FITC contained 130 nonspecifically fluorescent cells/100,000 cells analyzed, this analysis suggested that NKTa$^+$ cells occurred at a frequency of 0.19% in this individual. In several repeat experiments using either anti-NKTa or anti-NKTb, the range of NKTa$^+$ cells was found to be between 0.2 and 0.09% (mean 0.15% NKTa$^+$ cells).

As this low frequency of positive cells was very difficult to distinguish from nonspecific background fluorescence, we applied two adherence steps and two positive immune rosetting procedures with anti-NKTa antibody to enrich for cells that express the NKTa clonotype. As shown in Fig. 1, we were able to obtain a 20–50-fold enrichment using these procedures so that in several exper-

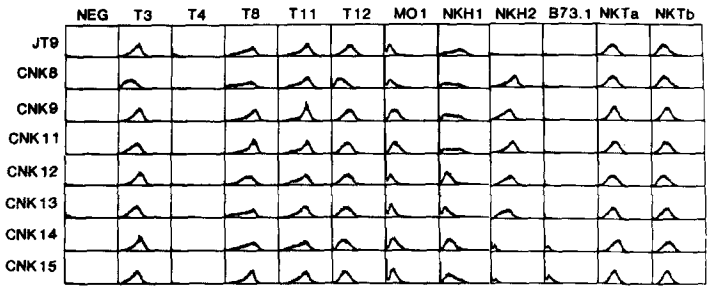

FIGURE 2. Surface phenotype of NKTa$^+$ cell lines. 10,000 cells were analyzed by indirect immunofluorescence and each histogram displays the number of cells (ordinate) vs. the intensity of fluorescence (abscissa) expressed on a logarithmic scale. Negative control used to determine background fluorescence was an ascites derived from a nonreactive hybridoma.

iments the level of reactivity was increased to 2.5–5% NKTa$^+$ cells. Indirect immunofluorescence analysis of the enriched cell population with anti-NKTa is shown in Fig. 1. Fig. 1*A* and region 1 in Fig. 1*B* clearly depict a discrete population of high density NKTa$^+$ cells, which in this experiment, represented 2.5% of 100,000 cells analyzed. The low density NKTa$^+$ population outlined in region 2 of Fig. 1*B* likely represents nonspecific binding of anti-NKTa. Therefore, we did not purify or characterize these cells in further experiments. An alternative possibility is that this low density NKTa$^+$ population is due to the modulatory effects of anti-NKTa during the overnight incubation period.

Direct Cloning of NKTa$^+$ Cells. In order to further characterize NKTa$^+$ cells in normal peripheral blood, we used immunofluorescent cell sorting to purify cells outlined in region 1 shown in Fig. 1*B*. NKTa-purified cells were then cloned into 800 wells using either autocloning or limiting dilution techniques, both at a plating frequency of 1 cell/well. After 3 wk of in vitro culture, 35 colonies were established and these were individually screened for killing of K562 target cells. 31 of 35 colonies (89%) were cytotoxic for K562 and were therefore felt to exhibit NK activity. Five of these clones could be established in long term culture and were used for further phenotypic and functional studies.

Generation of NKTa$^+$ Clones Following Purification of NKH2$^+$ Cells. In a separate series of experiments designed to generate NK clones with specific phenotypes, NKH2$^+$ cells were purified from normal nonadherent PBMC using immunofluorescent cell sorting on the Epics V. NKH2 antigen has previously been identified on ~50% of NK clones. However, large granular lymphocytes (LGL) in unstimulated peripheral blood that express NKH2 do not have a high degree of NK activity. When NKH2 sorted cells were purified from the same donor as clone JT9, cells were cloned by limiting dilution into 800 individual wells at a concentration of 1 cell/well using conditions identical to those described for generation of other NK clones. In this experiment, nine colonies could be expanded for further phenotypic and functional analysis. Each of these nine clones expressed NKH2 antigen and, interestingly, two clones, CNK8 and CNK9, also expressed the NKTa clonotype.

Phenotypic Characterization of NKTa$^+$ Clones. Fig. 2 shows the results of the phenotypic analyses of all new NKTa$^+$ clones. All clones were tested for expression of the NKTa clonotype as well as a number of other T cell and NK-

TABLE I
Cytotoxicity of NKTa$^+$ NK Cell Lines

Effectors	Targets						
	K562	REX	Molt-4	U937	Daudi	Laz 461	HL60
NKTa$^+$ clones							
JT9	83*	74	79	19	62	70	16
CNK8	87	65	81	2	70	24	13
CNK9	67	13	42	3	41	59	16
CNK11	75	48	42	3	53	71	23
CNK12	83	40	75	3	41	23	16
CNK13	81	47	70	27	61	23	33
CNK14	68	66	60	10	27	23	17
CNK15	82	72	79	12	39	77	18
NKTa$^-$ clones							
JT3	73	76	78	0	41	12	23
JT$_B$18	81	94	86	98	78	38	59
CNK6	81	83	88	5	27	18	30

* Percent specific cytotoxicity, SD ≤ 5%.

associated surface antigens. Moreover, the phenotype of CNK8 and CNK9, and the clones obtained from direct sorting of NKTa$^+$ cells, was compared with that of the previously established NKTa$^+$ clone, JT9. Previous reports (14–16) have shown that the phenotype of clones JT10 and JT11 is identical to that of JT9. As shown in Fig. 2, all clones express the clonotypic antigens NKTa and NKTb, and all are positive for antigens T3, T8, T11, T12, and Mo1. Similarly, the pan-NK cell antigen, NKH1, is expressed on all clones. All clones are negative for T4 and HNK1 (data not shown), but there are differences in the expression of NKH2. NKH2 is expressed on CNK8, CNK9, CNK11, CNK12, and CNK13, but not on JT9, JT10, JT11, CNK14, and CNK15. Thus, 50% of NKTa$^+$ clones express NKH2 to a large extent, and 50% do not express antigen detectable by indirect immunofluorescence.

Natural Killer Activity of NKTa$^+$ Clones. To evaluate the cytolytic activity of NKTa$^+$ clones obtained in various ways, we tested all clones against a series of seven different target cells. The cytotoxic specificity of all NKTa$^+$ clones is depicted in Table I and shows approximately equivalent cytotoxicity against this panel of targets. K562 and Molt-4 appear to be the most sensitive target cells and are uniformly killed by all clones. The target cell lines U937 and HL60 are quite resistant to cytotoxicity of NKTa$^+$ clones, although there is a low degree of killing in some instances. There is also a cytotoxic effect of all clones against the autologous EBV transformed B cell line Laz 461. Also shown in Table I is the cytotoxic activity of three NKTa$^-$,T3$^-$,NKH1$^+$ NK clones against the same targets. In these experiments, the NKTa$^+$ clones are shown to have a broad range of cytolytic activity that is similar to that seen for NK clones, CNK6 and JT3. In contrast, clone JT$_B$18 has more activity against targets, U937 and HL-60, which are resistant to lysis by NKTa$^+$ clones.

Inhibition of Cytotoxicity by mAbs, Anti-NKTa, and Anti-NKTb. Previous characterization of the NKTa antigen has shown that this structure is a 90-kD heterodimer that resolves into two distinct bands of 49 kD and 43 kD under

TABLE II

Inhibition of NK Activity of NKTa$^+$ Clones by Anti–NKTa and Anti–NKTb mAbs

Effectors	Targets											
	K562				Molt-4				Daudi			
	Medium	Anti-NKH1*	Anti-NKTa	Anti-NKTb	Medium	Anti-NKH1	Anti-NKTa	Anti-NKTb	Medium	Anti-NKH1	Anti-NKTa	Anti-NKTb
NKTa$^+$ clones												
JT9	82‡	79	7	33	61	58	6	7	66	61	5	48
CNK8	68	65	5	16	56	52	0	0	57	55	5	25
CNK9	75	71	5	15	32	30	0	0	66	62	6	36
CNK11	84	83	7	27	50	47	1	1	60	57	3	20
CNK12	53	48	13	41	68	64	0	0	31	27	0	17
CNK13	72	71	10	19	30	25	5	7	41	38	7	20
CNK14	57	54	4	16	25	23	0	0	21	20	1	11
CNK15	74	70	2	27	46	44	2	2	21	18	8	12
NKTa$^-$ clones												
JT3	64	62	61	63	76	76	76	77	31	30	28	28
JT$_B$18	68	63	72	63	73	71	76	76	62	62	58	61
CNK6	78	78	76	75	83	81	82	83	22	21	21	17

* Anti-NKH1 was used as a control antibody.

‡ Percent specific cytotoxicity; SD ≤ 5%; E/T ratio 5:1.

reducing conditions in SDS-PAGE. Anti-NKTb may identify a distinct epitope on the same cell surface structure, and both NKTa and NKTb antigens co-modulate with surface T3 antigen. NKTa antigen therefore appears to be analogous to T cell receptor-like structures (Ti) found on conventional T lymphocytes. To determine whether all NKTa$^+$ clones use this antigen as a surface receptor for target antigen, we assayed the ability of anti-NKTa to block cytotoxicity of all NKTa$^+$ clones. As shown in Table II, cytotoxicity of all NKTa$^+$ clones could be almost completely inhibited by anti-NKTa. Moreover, this high degree of inhibition was seen against three different target cells, K562, Molt-4, and Daudi. Anti-NKTb markedly inhibited cytotoxicity of all NKTa$^+$ clones when tested against Molt-4 targets, but significantly less inhibition was seen against either K562 or Daudi. Although the effect of anti-NKTb varied with different target cells, there was relatively little variation among the NKTa$^+$ clones. No inhibition could be observed when anti-NKH1 was added as a control antibody to the assay. As shown in Fig. 2, this antibody also binds to all NKTa$^+$ effector cells. As expected, there was no inhibitory effect against NKTa$^-$ NK clones such as JT3, JT$_B$18, and CNK6.

Inhibition of NKTa$^+$ Clones by Anti-TNK$_{TAR}$. To determine whether each NKTa$^+$ clone had similar specificity for TNK$_{TAR}$ antigen, we tested the ability of monoclonal anti-TNK$_{TAR}$ to block NK activity of each NKTa$^+$ clone. In addition, we tested 4F2 mAb, which has recently been shown to react with a different epitope on the same surface structure (27). Since it has previously been shown that these mAbs act at the target cell level, we preincubated target cells with either anti-TNK$_{TAR}$, 4F2, or both antibodies, and then added effectors. As shown in Fig. 3, there is a significant inhibition of cytotoxicity with either anti-TNK$_{TAR}$, 4F2, or the combination of both antibodies for each NKTa$^+$ clone. Both antibodies appear to have similar inhibitory activity and the combination of both antibodies does not in general increase the blocking of cytotoxicity. An

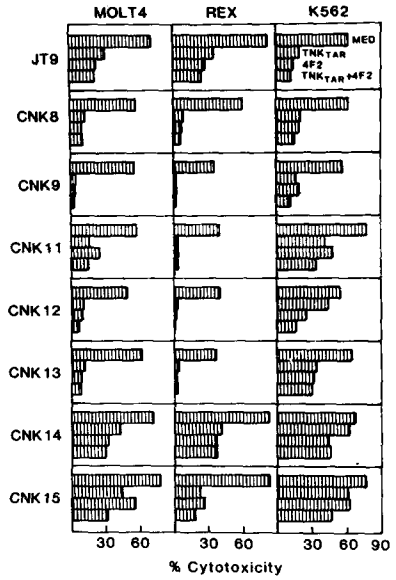

FIGURE 3. Cytotoxic activity of NKTa$^+$ clones after preincubation of the effector cells with either media, anti-TNK$_{TAR}$, 4F2, or a combination of both mAbs. Specific cytotoxicity was measured 4 h after addition of target cells as described in Materials and Methods. The cytotoxic assay was performed at an E/T ratio of 5:1.

mAb (5E9) directed against the transferrin receptor, another activation antigen expressed on rapidly dividing cells, had no inhibitory effect (data not shown).

Rearrangement of T Cell Receptor β Gene in NKTa$^+$ Clones. The *Ti* Cβ probe, which hybridizes equally well to the germline Cβ1 and Cβ2 segments of the gene encoding the T cell receptor, was used to analyze NKTa$^+$ clones for the presence of clonal *Ti β* gene rearrangements. Since not all restriction enzymes will provide detectable rearrangements, digestions with both Eco RI, which detects Cβ1 rearrangement, and Bam HI, which detects both Cβ1 and Cβ2 rearrangements (28), were performed for each cellular DNA preparation. After digestion with each restriction endonuclease, genomic DNA was size fractionated by agarose gel electrophoresis and hybridized with ^{32}P-labeled *Ti* Cβ REX probe. As shown in Fig. 4, all five different NKTa$^+$ clones that were tested had identical rearrangements following Bam HI digestion. The 23 kb band seen in the Bam HI digest (lanes *A–E*) and the 10.2 and 4.0 kb bands seen in the Eco RI digests (lanes *F–J*) represent the germline configuration of the *Ti β* genes. Each NKTa$^+$ clone contains an identical rearranged band of ~15 kb seen only in the Bam HI digests and not in the Eco RI digests. Since an Eco RI restriction site exists between the J and C regions of the Cβ2 locus (28), this finding suggests that all NKTa$^+$ clones have an identical rearrangement of Cβ2 and are likely to have a deletion of one of the Cβ1 genes, whereas the second allele remains in germline configuration.

Control experiments in Fig. 5 show that the Cβ2 rearrangement of CNK9, CNK11, CNK12, CNK14, and CNK15 shown in Fig. 4 is identical to that previously shown for JT9 (24). In contrast, CNK3 cells (lane *H*) have a Cβ1 rearrangement, visible in the Eco RI digest. For comparison, Laz 461 (lanes *A* and *D*) contains both alleles in the germline configuration. The possible deletion

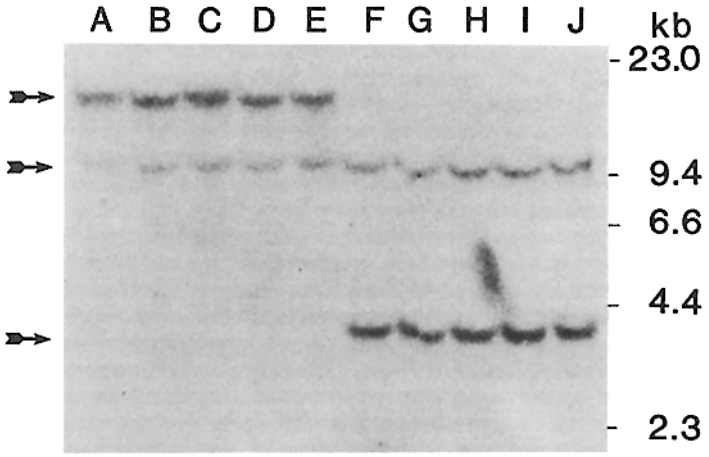

FIGURE 4. Ti β gene rearrangements in NKTa$^+$ clones. Southern blot analysis of DNA from five clones was digested with either Bam HI (lanes *A–E*) or Eco RI (lanes *F–J*) and hybridized with ^{32}P-labeled Ti Cβ probe. Lanes contained approximately 10 μg of DNA from different clones as follows: CNK9 (lanes *A* and *F*); CNK11 (lanes *B* and *G*); CNK12 (lanes *C* and *H*); CNK14 (lanes *D* and *I*); CNK15 (lanes *E* and *J*).

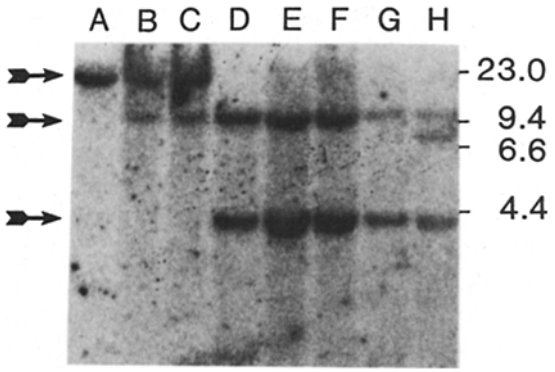

FIGURE 5. Southern blot analysis of NKTa$^+$ clones compared with T3$^+$NKTa$^-$ clone and EBV-induced B cell line. DNA from NK clones or germline Laz 461 was digested with either Bam HI (lanes *A–C*) or Eco RI (lanes *D–H*) and hybridized with ^{32}P-labeled Ti Cβ REX probe. Lanes contained ~10 μg of DNA from different clones as follows: Laz 461 (lanes *A* and *D*); CNK11 (lanes *B* and *E*); CNK12 (lanes *C* and *F*); JT9 (lane *G*); CNK3 (lane *H*).

of one of the Cβ1 alleles in each NKTa$^+$ clone is supported by the finding that the 10.2-kb band seen in the Eco RI digests is consistently less intense than the germline 4.0-kb fragment (Fig. 4). In Laz 461 cells that have neither rearrangement nor deletion of Cβ1 (Fig. 5, lane *D*), both fragments are of similar intensity.

Discussion

Previous studies have described the characterization of three different NK clones, JT9, JT10, and JT11, derived from the same individual. Each of these clones was found to have a mature T cell phenotype and expressed the same clonotypic T cell receptor-like structure that was identified with two different mAbs, anti-NKTa and anti-NKTb. In subsequent experiments, anti-NKH2 an-

tibody, which defines a subset of LGL in normal peripheral blood, was used to establish NK clones that express this antigen. Analysis of the NKH2$^+$ clones which resulted from these experiments revealed that two of these, CNK8 and CNK9, also had a mature T cell phenotype, and both clones expressed the same NKTa clonotypic receptor-like structure. As expected, both CNK8 and CNK9 clones expressed NKH2 antigen, but this antigen was not expressed in the three original NKTa$^+$ clones JT9, JT10, or JT11. This finding thus suggested that different NK clones (i.e., NKH2$^-$ and NKH2$^+$ clones) might use the same cell surface receptor for antigen (NKTa). Therefore, further studies initially focused on the identification of NKTa$^+$ cells in unstimulated peripheral blood from the same individual from whom NKTa$^+$ clones had been generated. Using flow cytometric analysis, we were able to show a frequency of NKTa$^+$ cells in peripheral blood of ~0.15%. Using two adherence steps and two positive immune rosetting procedures, we were able to achieve a 20–50-fold enrichment up to 2.5–5% NKTa$^+$ cells. Flow cytometric immunofluorescent cell sorting was then used to purify a discrete NKTa$^+$ cell population and to generate an additional series of NKTa$^+$ clones based primarily on the surface expression of this clonotypic antigen. Of the 35 colonies established in this manner, 89% exhibited NK activity when screened for killing of K562 target cells. Five of these NK active clones were then expanded in vitro for phenotypic and functional characterization and for comparison with other NKTa$^+$ clones.

The phenotypic comparison of the five new NKTa$^+$ clones with CNK8 and CNK9, and the previously established JT9 clone confirmed that all clones expressed the NKTa clonotype and were also positive for NKTb, a second antigenic determinant on the same T cell receptor-like structure. In addition, all clones express the T cell antigens T3, T8, T11, and T12, the myelomonocytic antigen Mo1, and the pan-NK cell antigen NKH1. In contrast, NKH2, an antigen that is normally expressed on a small subset of peripheral blood LGL, was only expressed on half of the NKTa$^+$ clones. Interestingly, this included all clones selected for expression of NKH2 (CNK8 and CNK9) and three of five clones selected for expression of the NKTa clonotype, but none of the original clones selected on the basis of functional NK activity (JT9, JT10, JT11). Since the expression of NKH2 antigen has been found to be quantitatively stable and cell cycle independent during prolonged in vitro culture of NK cells, the difference in NKH2 antigen expression among NKTa$^+$ clones suggests that these cells represent distinct clonal populations.

With regard to the cytotoxic function of NKTa$^+$ cells, it is important to note that 89% of colonies selected solely on the basis of expression of NKTa clonotype displayed cytotoxic activity against K562. Subsequently, the evaluation of cytolytic activity of all NKTa$^+$ clones against seven different targets demonstrated similar levels of cytotoxicity that were comparable to other NKTa$^-$ NK clones. For both NKTa$^+$ and NKTa$^-$ clones, cytolytic activity does not appear to be MHC restricted since targets that lack either MHC class I or class II antigens are killed equally well.

Since the NKTa antigen has been defined as a T cell receptor-like structure, it was of interest to determine whether all NKTa$^+$ clones used this antigen for target cell recognition. Cytotoxicity inhibition studies of NKTa$^+$ clones showed

that the addition of monoclonal anti-NKTa antibody abrogated cytotoxic activity of all $NKTa^+$ clones almost completely. Although anti-NKTb did not block cytotoxicity against all targets, the differential blocking of anti-NKTb was found to be consistent among all $NKTa^+$ clones. In contrast, cytotoxicity of none of the $NKTa^-$ clones could be blocked by either mAb against this clonotypic antigen. Further analysis of the specificity of $NKTa^+$ clones at the target cell level indicated that all clones are specific for the same 140-kD activation antigen on the target cell, termed TNK_{TAR}. In these experiments, preincubation of target cells with anti-TNK_{TAR} or 4F2 mAb, or a combination of both markedly inhibited cytotoxicity of $NKTa^+$ clones, whereas $NKTa^-$ NK clones were not affected (data not shown). Taken together, these results provide strong evidence that the cytotoxic specificity of $NKTa^+$ clones is conferred by the NKTa structure.

Since a small population of peripheral blood lymphocytes appear to use the same NKTa structure for target cell recognition despite expression of at least two different phenotypes, it was important to determine whether these phenotypically distinct clones would express identical T cell receptor gene rearrangements. Using the *Ti* Cβ REX probe, it was found that each $NKTa^+$ clone contains an identical rearranged band of ~15.0 kb in the Bam HI digests, and no rearrangements were present in the Eco RI digests. These data therefore indicate that all $NKTa^+$ clones have an identical *Ti* β gene rearrangement using Cβ2 and not Cβ1. It can therefore be presumed that a functionally rearranged Cβ2 gene (VDJC) plays a role in determining the MHC independent specificity of these $NKTa^+$ NK clones for TNK_{TAR} antigen. *Ti* α rearrangements could not be evaluated in this series of NK clones, and the possibility remains that relatively minor differences in their specificity may be due to different rearrangements of the *Ti* α subunit.

These results with $NKTa^+$ clones contrast sharply with recent reports that have described the expression of other clonotypic T cell receptor-like structures present in low percentages (~2%) in peripheral blood of normal individuals (29–31). In these other studies, the functional and molecular analysis of T cell receptor gene expression in T cell lines and T cell clones has indicated that T cell clones expressing the same clonotypic *Ti* β variable (V) gene product can express either T4 or T8 antigen and can exert inducer, suppressor, and/or cytotoxic function. Moreover, this analysis indicated that individual members of the V gene family could not be linked to *Ti* diversity (D) and/or joining (J) and constant (C) region segments and that Ti α chains of such clones were also distinct. The implications of these findings for conventional MHC-restricted T cells is that the use of a single *Ti* β V gene is not restricted to any functionally or phenotypically defined population (31). In contrast, the NKTa clonotype appears to be very closely linked in all NK clones to the non–MHC-restricted cytotoxic specificity for the TNK_{TAR} antigen. Thus, the NKTa structure may be defined by a more complex VDJ region on *Ti* β and this region may be critical in determining the MHC-independent specificity of NK active clones with a mature T cell phenotype.

Taken together, we can conclude from our studies that the NKTa clonotype defines a distinct population of peripheral blood cells. This raises at least two major questions. First, is this clonotypic antigen unique for one individual or can

it be found in other individuals as well? Since the $NKTa^+$ population is very small, this question cannot be easily answered by immunofluorescent analyses, even if immune rosetting is used to enrich for these cells. Thus, in vitro stimulation and propagation of clonotype positive cells from other individuals and clonal expansion will be necessary to evaluate the functional use of this antigen in the general population. The second question concerns the functional role of the NKTa antigen in the individual we have studied. Since NK active $NKTa^+$ clones with different phenotypes have been derived from the same individual at various times over a period of 3 yr, it is unlikely that such cells would exist primarily to control tumor cell growth, or that they are present in response to a specific infectious agent. The finding that $NKTa^+$ cells specifically recognize a target antigen (TNK_{TAR}) that is broadly expressed on early hematopoietic cells and activated cells suggests that $NKTa^+$ effector cells may have an immunoregulatory function in vivo. Further studies are in progress to evaluate the role of TNK_{TAR} and the involvement of $NKTa^+$ cells in such functions as the regulation of hematopoietic differentiation, B cell stimulation and differentiation, as well as in the regulation of NK cell function itself.

Summary

Over a period of 3 yr, a series of ten NK clones that express a unique clonotypic T cell receptor-like structure, termed NKTa, has been generated from a single individual. These clones were derived from either peripheral blood nonadherent cell fractions (JT9, JT10, JT11), NKH2-purified cells (CNK8, CNK9), or NKTa-purified cells (CNK11, CNK12, CNK13, CNK14, CNK15). Flow cytometric analysis of peripheral blood mononuclear cells from this individual showed that $NKTa^+$ cells occur with a frequency of ~0.15%. The existence of $NKTa^+$ cells in peripheral blood was confirmed by use of immunorosette enrichment techniques, flow cytometric purification, and subsequent clonal expansion of $NKTa^+$ cells. Phenotypic analysis of $NKTa^+$ clones showed that all expressed NKH1 as well as T3, T8, T11, T12, and Mo1 antigens. Only five of ten clones expressed NKH2 antigen. All $NKTa^+$ clones had broad cytolytic activity against a series of seven different target cells that was similar to that of other NK clones. In addition, cytotoxicity of each clone could be inhibited by preincubation of effector cells with monoclonal anti-NKTa or by preincubation of target cells with monoclonal anti-TNK_{TAR}. Although half of the $NKTa^+$ clones appeared phenotypically different from the other half with regard to the expression of NKH2 antigen, analysis of T cell receptor gene rearrangements indicated that all $NKTa^+$ clones contained identical gene rearrangements of $C\beta 2$.

Received for publication 10 October 1985 and in revised form 10 December 1985.

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