IDENTIFICATION OF A CLONALLY RESTRICTED 90 kD HETERODIMER ON TWO HUMAN CLONED NATURAL KILLER CELL LINES

Its Role in Cytotoxic Effector Function*

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Recent studies in both human and murine systems have suggested that natural killer (NK)[1] activity can be mediated by different populations of effector cells (1–3). In human peripheral blood, most NK cells were found in the null cell fraction (4) and were morphologically identified as large granular lymphocytes (LGL) (5). When analyzed with monoclonal antibodies directed at well-defined surface structures, purified LGL were shown to be a heterogeneous population of cells, probably containing various subsets. For example, 30% of LGL react with anti-T8, 70–80% with anti-T11 and/or anti-Mo1, and 40–50% with anti-T10 (6–8). In addition, many studies have demonstrated that not only LGL but also activated T lymphocytes were able to mediate NK or NK-like activity (9–11).

To both dissect the heterogeneity of NK cells and subsequently perform studies with homogeneous populations of these cytotoxic lymphocytes, we have recently developed human NK clones (7). This approach was made possible by previous studies which demonstrated that NK cells can be cultured and expanded in conditioned medium containing interleukin 2 (IL-2), and that cultured cells maintain stable phenotype and functional activity for prolonged periods of time in vitro (12, 13). Furthermore, when a series of human NK clones was analyzed with monoclonal antibodies, their phenotypic heterogeneity confirmed previous observations made with uncloned populations of cells (14). Three groups of clones could be distinguished by analysis of cell surface markers. One group was

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[1] *Abbreviations used in this paper*: CTL, cytotoxic T lymphocytes; EBV, Epstein-Barr virus; E/T, effector/target; F/H, Ficoll-Hypaque; IL-2, interleukin 2; PHA, phytohemagglutinin; LCM, lymphocyte-conditioned medium; LGL, large granular lymphocytes; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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shown to phenotypically represent mature T lymphocytes. A second group, derived from purified LGL, expressed distinct combinations of T lymphocyte-associated surface structures but lacked the mature T cell differentiation antigen T3. Finally, one clone did not express any T cell markers or any other lineage-related differentiation antigens. Although NK clones were obtained using a variety of initial triggering stimuli for proliferation, they were all selected on the basis of the same functional criteria, i.e., the capacity to lyse K562 cells that are used as a standard target in NK assays. In addition, when these clones were tested in cytotoxicity assays against a panel of established cell lines, it was found that these clonal populations of lymphocytes had distinct target specificity (14). Taken together, these results suggested that what is usually referred to as NK or NK-like activity (i.e., the ability to kill K562 cells without apparent presensitization in vitro) is mediated by populations of lymphocytes that have distinct phenotypes and functional repertoires.

The present studies are directed at identifying the structural basis of effector/target cell interaction for individual human NK clones. This report describes experiments performed with two NK cloned cell lines termed JT9 (14) and JT10 (8), which were both independently derived from phytohemagglutinin (PHA)-stimulated lymphocytes of a single donor and selected initially for their ability to kill K562 cells. Through the generation of a monoclonal antibody produced against JT9, it was found that both JT9 and JT10 cells, which have a mature T lymphocyte phenotype ($T1^+T3^+T8^+T11^+T12^+$), express a 90 kD heterodimeric structure. This structure, termed NKTa, is linked to the T3 molecule and appears to be analogous to Ti clonotypic determinants (15) that have been identified on specific alloreactive cytotoxic T lymphocytes (CTL). Our studies suggest that the NKTa molecule present on JT9 and JT10 cells is intimately involved in determining the target specificity of these two NK active clones.

Materials and Methods

Isolation of Subpopulations of Peripheral Blood Cells. Human peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteer donors by Ficoll-Hypaque (F/H) density gradient centrifugation. Monocyte-enriched adherent cells were obtained from PBMC by two-step adherence on plastic culture dishes. Granulocytes were isolated from F/H gradient pellets using high molecular weight dextran (8). Activated T cells were generated by stimulating PBMC with either PHA (2 μg/ml), allogeneic Epstein-Barr virus (EBV)-transformed B cells (Laz 388), or autologous EBV-transformed B cells (Laz 401) at a 1:1 stimulator/responder ratio. They were tested on day 6 after stimulation.

Previously Characterized Monoclonal Antibodies. Monoclonal antibodies used in these studies have been previously described in detail. T1 and T12 antigens are expressed on a fraction of thymocytes and on the vast majority of peripheral blood T cells (16). Anti-T3 defines all mature T lymphocytes in human peripheral blood (17). T8 antigen is expressed on a subset of peripheral T cells having primarily cytotoxic/suppressor function (18). Functional properties of anti-$T8_{A,B,C}$ have been previously described in detail (19). Anti-T11 defines an antigen associated with the sheep erythrocyte receptor (16). In peripheral blood, 901 antigen is expressed on ~7% of cells that morphologically appear to be a homogeneous population of LGL. >95% of NK activity in peripheral blood is found within the purified 901^+ fraction. Anti-901 antibody was found to be reactive with 1% of $T3^+$ cells by fluorescence microscopy but does not react with monocytes, granulocytes, B cells, erythrocytes, or platelets (8).

Generation of Human Cloned Cell Lines. Methods for generation of human NK cloned

cell lines and allogeneic CTL clones have been described in detail (7, 14). Briefly, clones were obtained using a limiting dilution technique. Either PBMC or LGL were cloned at one cell per well on a feeder layer of autologous, irradiated (5,000 rad) PBMC plus either PHA (2 μg/ml) or allogeneic or autologous, irradiated (5,000 rad) EBV-transformed B cells. Selected colonies were expanded by the addition of culture medium containing lymphocyte-conditioned medium (LCM) (10–15% final dilution) every 3 d. Culture medium was RPMI 1640 supplemented with 1% penicillin-streptomycin, 1% sodium pyruvate, and 20% human AB serum. All cell lines used in these studies have been subcloned at least four times at 100 cells per well on a feeder layer of autologous, irradiated PBMC plus irradiated EBV-transformed B cells. After subcloning procedures, both phenotype and cytotoxic function have remained stable.

Preparation of LCM Containing IL-2. LCM was produced by stimulating whole PBMC at a concentration of 2.5×10^6/ml for 2 h with 5 μg/ml PHA (Burroughs Wellcome & Co., Greenville, NC), 5 ng/ml phorbol myristate acetate (Sigma Chemical Co., St. Louis, MO), and 5,000 rad-irradiated Laz 388 (0.5×10^6/ml). The cells were then washed four times to remove the mitogens and were resuspended in RPMI 1640 supplemented with 2.5% human AB serum. After 40 h of incubation at 37°C, the supernatants were harvested, passed through 0.45 μm filters, and stored at −70°C.

Production of the Anti-NKTa Antibody. 6-wk-old female BALB/c mice were immunized with JT9 cells. The immunization schedule consisted of an initial intraperitoneal injection of 2×10^6 cells emulsified in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, MI) followed by two weekly intraperitoneal injections of 2×10^6 viable cells in phosphate-buffered saline (PBS). 2 wk later, 2×10^6 cells were injected intravenously, followed by splenectomy 3 d later. Somatic cell hybridization was carried out by the method of Kohler and Milstein (20).

Cell Lines. Several continuously growing cell lines were used in these studies. Laz 461, Laz 156, and Laz 388 are EBV-transformed lymphoblastoid B cell lines. Nalm-1 and Laz 221 are common acute lymphoblastic leukemia antigen (CALLA)-positive acute lymphocytic leukemia cell lines. Daudi was derived from a Burkitt's lymphoma. Molt-4, CEM, HPB ALL, JM, and HSB are T cell leukemia cell lines. K562 was established from a patient with chronic myelogenous leukemia and HL60 from a patient with acute promyelocytic leukemia. U937 is a histiocytic cell line.

Cytotoxicity Assays. To investigate the functional effects of monoclonal antibodies, cloned cells were incubated for 18 h with antibodies or medium before the addition of ^{51}Cr-labeled targets. Cytotoxic activity of the clones was tested in 4-h assays. All experiments were performed in triplicate using V-bottomed microtiter plates according to a standard method previously described (8, 14). Medium for cytotoxic assays was RPMI 1640 plus 20% human AB pooled serum and 1% penicillin-streptomycin. Assays were performed at a 10:1 effector/target (E/T) ratio using 4,000 target cells per well.

Proliferative Studies. To investigate the effects of monoclonal antibody treatment on their proliferative capacity, 25,000 cloned cells were incubated for 24 h with saturating amounts of LCM containing IL-2 and/or saturating amounts of antibody in round-bottomed microtiter plates (Costar, Cambridge, MA). Experiments were done in culture medium. Cultures were then pulsed for 12–18 h with 0.2 μCi of tritiated thymidine and subsequently harvested on a MASH II apparatus (Microbiological Associates, Walkersville, MD). Tritiated thymidine incorporation was measured using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

Modulation of Surface Antigens by Monoclonal Antibodies. Cloned cells at 1×10^6/ml were incubated with saturating amounts of one or another monoclonal antibody for 36 h at 37°C in final culture media. Control cells were cultured in media alone. Subsequently, the cells were washed three times and analyzed for expression of cell surface antigens by means of indirect immunofluorescence assays performed at 4°C.

Phenotypic Analysis of Cell Surface Antigens. Phenotypic analysis was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse Fab IgG as described previously (7). Samples were analyzed on an Epics V flow cytometer (Coulter Electronics, Hialeah, FL). 10,000 cells were analyzed in each sample. Each histogram

displays the number of cells (ordinate) versus the intensity of fluorescence (abscissa) expressed on a logarithmic scale. The negative control used to determine background fluorescence was an ascites derived from a nonreactive hybridoma. Monoclonal antibodies were always used at saturating concentrations (1:100–1:500).

^{125}I Surface Labeling and Immunoprecipitation of Antigens from JT9 Cell Line. Cells were surface labeled with ^{125}I (New England Nuclear, Boston, MA) using a standard lactoperoxidase method previously described (15). 2×10^7 viable, proliferating cells were washed with RPMI 1640, resuspended in PBS, and labeled with 1 μCi ^{125}I for 15 min at room temperature, followed by the addition of 100 μl NaI (1 mol/liter). Labeled cells were then harvested and washed three times in cold RPMI 1640. Cell lysate was prepared by resuspending the cell pellet in 300 μl RIPA buffer, pH 7.5, containing 1% Triton X-100, 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and trypsin inhibitor and agitated for 40 min at 4°C. Cell lysates were stored at −70°C before use.

Immune precipitations were carried out using preformed complexes of rabbit anti-mouse Ig and specific monoclonal antibody as described previously (21). Briefly, macromolecular aggregates were removed from cell lysate by ultracentrifugation for 30 min at 100,000 *g*. Cell lysates were then precleared three times by incubation at 4°C for 1 h with either formalin-fixed *Staphylococcus aureus* bacteria and/or preformed complexes of rabbit anti-mouse Ig and nonspecific monoclonal antibody. Aliquots of precleared lysate were then incubated with specific preformed complexes for 2 h at 4°C. Immune precipitates were then washed four times with RIPA buffer as above and then dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and loaded onto separate slots on a 10% polyacrylamide slab gel. Identical aliquots of immune precipitates were run in reduced and nonreduced conditions. Gels were dried and radiolabeled precipitates were visualized using standard methods (22).

Results

Generation of a Monoclonal Antibody Blocking Cytotoxic Activity of JT9 Cells. After immunization of mice with JT9 cells, 1,250 hybridoma colonies were produced in a series of five fusion experiments. Hybridoma supernatants were primarily screened for their ability to block the cytotoxicity of the JT9 clone towards K562 cells. For this purpose, constant numbers of cloned cells were incubated overnight with individual supernatants, and cytotoxic activity was subsequently measured using ^{51}Cr-labeled K562 targets. One hybridoma that totally blocked cytotoxicity of JT9 cells was selected for further studies. It was recloned twice by limiting dilution and immune ascites was produced. This monoclonal antibody (termed anti-NKTa) was of the IgG1 subclass as determined by Ouchterlony immunoprecipitation using standard reagents. Its activity was then retested under the same experimental conditions used for the initial screening. Anti-T11 and anti-T12, previously shown to be both reactive with JT9 cells and unable to block their cytotoxic function, were used as negative controls. Results presented in Fig. 1 are representative of five individual experiments and show that anti-NKTa totally abrogated the cytotoxicity of the clone. Blocking of cytotoxicity was concentration dependent and the optimal blocking effect was reached at a 10^{-3} dilution of the ascites. This dilution was then used in subsequent assays. Viability of JT9 cells as determined by trypan blue dye exclusion was not affected by incubation with anti-NKTa ascites at any dilution.

JT9 cells were previously shown to proliferate in LCM containing IL-2 (14). This proliferative response was tested in the presence of the anti-NKTa antibody in order to exclude the possibility that the inhibition of cytotoxicity produced by

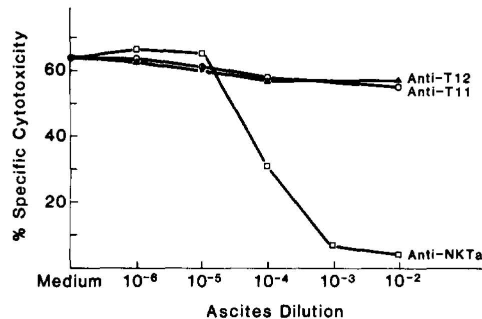

FIGURE 1. Inhibition of the cytotoxicity of JT9 cells towards K562 cells by anti-NKTa monoclonal antibody. Mean ± SD ≤ 5% for each point; E/T ratio, 10:1.

TABLE I
Influence of Monoclonal Antibodies on Proliferative Response of JT9 Cells to IL-2

	Untreated	Anti-$T8_A$	Anti-T3	Anti-NKTa
Medium	72 ± 26*	56 ± 6	45 ± 22	288 ± 42
IL-2‡	1,320 ± 93	1,245 ± 21	4,034 ± 262	4,826 ± 348

* Results are expressed as mean counts per minute for triplicate cultures ± SD.
‡ LCM containing IL-2.

this reagent could be attributed to a general decrease in the function of the cloned cells. The influence of anti-T3 and anti-$T8_A$ antibodies was tested in parallel. JT9 cells were cultured for 36 h with saturating concentrations of each monoclonal antibody in the presence of IL-2. [^{3}H]thymidine incorporation was measured during the last 12 h of culture. It was found that both anti-T3 and anti-NKTa strongly increased the proliferative response of the clone to IL-2, whereas anti-$T8_A$ had no effect (Table I).

Cellular Distribution of the NKTa Antigen. The reactivity of the anti-NKTa antibody was tested on purified subpopulations of lymphoid and nonlymphoid cells by indirect immunofluorescence assay and subsequent analysis using an Epics V. As shown in Table II *A*, all the cellular populations studied, either autologous to JT9 or derived from other donors, were found to be negative. In addition, a series of in vitro established cell lines, including those used as targets in cytotoxicity assays, were shown to be unreactive with anti-NKTa antibody (Table II *B*). The expression of the NKTa antigen was subsequently investigated using several cloned cell lines displaying distinct phenotypes and cytotoxic functions (Table II *C*). JT1 and JT3, previously described NK clones (14), were negative, as were JT12, 13, and 14, which are three specific alloreactive CTL clones derived from the same donor and directed to the EBV-transformed cell line Laz 388. Five additional and well-characterized CTL clones, including the $CT8_{III}$ line (15) derived from a different donor, were also found to be unreactive (data not shown).

In contrast, one NK cloned cell line (in addition to JT9) termed JT10 did express the NKTa antigen. JT10 was obtained in cloning experiments performed 6 mo after those in which JT9 was produced. Samples of the initial clone were frozen away immediately after screening procedures, and the presence of the

TABLE II

Screening for Reactivity of the Anti-NKTa Antibody

A. Cell fractions*	
Autologous cells‡§	Allogeneic cells
Peripheral blood mononuclear cells	Peripheral blood mononuclear cells (3)‖
Monocytes	Monocytes (3)
Granulocytes	Granulocytes (3)
Red cells	Red cells (2)
PHA-activated T cells,	PHA-activated T cells (3)
Alloreactive T cells	
Autoreactive T cells¶	Thymocytes (2)

B. In vitro established tumor- or viral-transformed cell lines*§

B cells: Laz 221, Nalm-1, Daudi, Laz 388, Laz 156
T cells: Molt-4, CEM, HSB, HPB-ALL, JM
Nonlymphoid cells: K562, KG-1, U937, HL60

C. Cloned cytotoxic cell lines§	Phenotype	Function	NKTa reactivity
JT1	$T3^-T4^-T8^-T11^-901^+$	NK	—*
JT3	$T3^-T4^-T8^-T11^+901^+$	NK	—
JT9	$T3^+T4^-T8^+T11^+901^+$	NK	>95
JT10	$T3^+T4^-T8^+T11^+901^+$	NK	>95
JT12	$T3^+T4^-T8^+T11^+901^-$	CTL	—
JT13	$T3^+T4^-T8^+T11^+901^-$	CTL	—
JT14	$T3^+T4^-T8^+T11^+901^-$	CTL	—

* Number of positive cells was <4% in each determination unless otherwise mentioned.
‡ Cells autologous to JT9.
§ Two or more determinations per cell fraction or cell line.
‖ Number of individuals tested.
¶ Autologous T cells stimulated by autologous EBV-transformed B cells.

NKTa antigen was found on both initially frozen cells and on those maintained in long-term cultures. The origin of JT10 is similar to that of JT9 since it is derived from PBMC (of the same individual) initially stimulated by PHA. The two clones have identical phenotypes when assessed using anti-T1, -T3, -T4, -T8, -T11, -T12, and -901 antibodies.

Evidence for Comodulation of NKTa and T3 Structures. A series of experiments was performed to assess whether anti-NKTa antibody would induce modulation of its target antigen. JT9 cells were cultured for 24 h at 37°C in the presence of a saturating concentration of anti-NKTa. Cells were subsequently washed and analyzed for expression of NKTa, T3, T8, T11, and T12 antigens using an indirect immunofluorescence assay at 4°C. As shown in Fig. 2, it was found that anti-NKTa antibody was able to induce modulation of its own target structure. In addition, incubation with anti-NKTa at 37°C resulted in the simultaneous decrease in expression of the surface T3 molecule. In contrast, the expression of T8 antigen was not modified. Other surface antigens such as T11 and T12 were also not affected when modulation was induced by anti-NKTa antibody (data not shown). Reciprocal experiments were conducted in which JT9 cells were incubated with anti-T3 for 24 h. When cells were reanalyzed, it was found that as a result of the modulation induced by anti-T3 (16), the expression of both T3 and NKTa antigen was markedly decreased whereas expression of T8

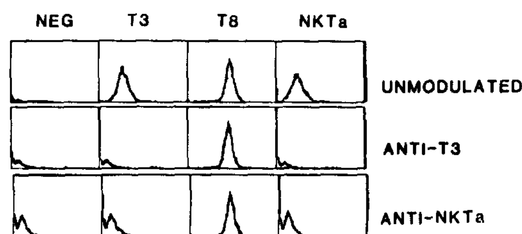

FIGURE 2. Co-modulation of T3 and NKTa antigen. JT9 cells were divided into three groups (vertical axis) and incubated for 24 h with medium (unmodulated), anti-T3, or with anti-NKTa antibody. After this period of culture, cells from each group were washed three times and retested at 4°C for expression of T3, T8, and NKTa antigens (horizontal axis) using an indirect immunofluorescence assay. Each population was then analyzed using an Epics V flow cytometer. Anti-T6 antibody was used as a negative control (NEG) in these experiments.

TABLE III

Influence of Anti-T3, Anti-NKTa, and Anti-T8 Antibodies on Cytotoxic Activity of JT9 Cells

Preincubation	Target cells										
	K562	KG-1	U937	Daudi	Laz 221	Nalm-1	Molt-4	HSB	HPB ALL	JM	CEM
Medium	70*	79	44	85	61	75	76	62	58	76	62
Anti-T3	20	11	6	2	4	10	14	37	4	33	10
Anti-NKTa	12	13	2	1	2	2	2	25	2	8	8
Anti-$T8_A$	59	92	51	91	65	99	76	80	53	76	63

* Percent specific cytotoxicity, SD ≦ 5%; E/T ratio, 10:1.

antigen was not affected (Fig. 2).

Influence of Anti-NKTa, T3, and T8 Antibodies on the Cytotoxicity of JT9 Cells Towards a Panel of Sensitive Targets. It has been previously demonstrated that the JT9 clone, although initially selected for its ability to kill K562 cells, is also cytotoxic for a variety of unrelated tumor cells (14). The blocking capacity of anti-NKTa, -T3, and -T8 antibodies could therefore be assessed using a large panel of sensitive target cell lines. As shown in Table III, after 18 h of preincubation with either anti-NKTa or anti-T3, the cytotoxicity of JT9 cells towards all targets tested was strongly decreased. In contrast, anti-$T8_A$, which blocks the cytotoxicity of $T8^+$ alloreactive T cell clones (19), did not influence the lytic capacity of JT9 cells against any target. Anti-$T8_B$, anti-$T8_C$, and an additional anti-T8 antibody obtained after immunization with JT9 cells were also tested using a more restricted panel of target cells. None of these anti-T8 antibodies were able to block the cytotoxic function of JT9 cells (data not shown). Additional control experiments not included in Table III indicated that anti-NKTa was not able to block cytotoxicity of either NK or CTL clones found to be unreactive with the antibody in immunofluorescence assays.

Immunoprecipitation of ^{125}I-labeled NKTa Antigen. Immunoprecipitation experiments using preformed complexes of rabbit anti-mouse IgG and NKTa antibody and ^{125}I surface-labeled JT9 cell lysates were performed as described in Materials and Methods. As shown in Fig. 3, anti-NKTa precipitated two bands with molecular masses of approximately 49,000 and 43,000 daltons under reducing conditions in SDS-PAGE. Under nonreducing conditions, this antigen appears

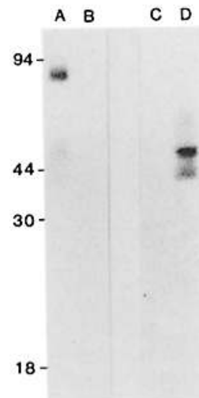

FIGURE 3. Immunoprecipitation of NKTa antigen from ^{125}I surface-labeled JT9 cells. Immunoprecipitation of ^{125}I-labeled JT9 cells was resolved by SDS-PAGE using 10% polyacrylamide gels as described in Materials and Methods, under both reduced and nonreduced conditions. Radiolabeled standards of various molecular weights were run in parallel with immunoprecipitates as follows: (*A*) anti-NKTa, nonreduced, (*B*) negative control complex (anti-MY4), nonreduced, (*C*) negative control complex (anti-MY4), reduced, (*D*) anti-NKTa, reduced.

as a single band corresponding to a molecular mass of 90 kD. Similar results were obtained using [^{35}S]cysteine metabolically labeled JT9 cells (data not shown).

Identical Reactivity of JT9 and JT10 Cells Towards a Panel of Randomly Selected Target Cells. Immunoprecipitation experiments indicated that anti-NKTa antibody was directed at a heterodimer of similar molecular weight as that of Ti clonotypic determinants recently identified on specific CTL clones (15, 23). Since anti-Ti antibodies blocked antigen-specific proliferation of CTL clones as well as cytotoxicity against antigen-bearing target cells (15, 23), it has been postulated that Ti determinants were likely to represent antigen-receptor structures on specific T lymphocytes. The role of NKTa molecules present on JT9 and JT10 cells was analyzed in light of these previous results. Since it has been established that in humans, individual NK clones can have different repertoires for target cell recognition and/or lysis (14), it was of particular interest to assess whether two independently derived NK clones that were found to express the same 90 kD clonotype would have identical or distinct target specificity. For this purpose, 15 in vitro established cell lines were randomly selected because of their availability in the laboratory and used as target cells in the NK assays. One previously described clone, termed JT3, was used as a control in these experiments. As shown in Table IV, JT9 and JT10 cells were found to display a superimposable pattern of reactivity towards the panel of cell lines tested. Results obtained in these assays were rather clearcut. Indeed, specific cytotoxicity of JT9 and JT10 cells ranged from 45 to 100% for one group of target cells, but was <10% for the other. The reactivity of the JT3 clone was distinct from that of JT9 and JT10 since, for example, JT3 cells did not exhibit significant lysis for target cells such as Nalm-1.

JT9 Cells Do Not Interact with Target Cells Via Recognition of Class I or Class II Products of the Major Histocompatibility Complex (MHC) Region. The experiments reported above strongly suggested that JT9 and JT10 cells use a T3-90 kD

TABLE IV
Cytolytic Activity of JT3, JT9, and JT10 Cloned Cells

Effector cells	Targets							
	K562	KG-1	U937	HL60	Daudi	Laz 221	Nalm-1	Laz 461
JT3*	+‡	+/−	+/−	−	+	+/−	−	−
JT9	+	+	+	−	+	+	+	−
JT10	+	+	+	−	+	+	+	−
	Laz 156	Laz 388	Molt 4	HSB	HPB-ALL	JM	CEM	
JT3*	−	−	+	+	+/−	+	+/−	
JT9	−	−	+	+	+	+	+	
JT10	−	−	+	+	+	+	+	

* Results are representative of at least three individual experiments for each clone.
‡ Specific cytotoxicity: +, ≧45%; +/−, <45%; −, <10%. E/T ratio, 10:1.

TABLE V
Influence of Anti-HLA and Anti-Ia Antisera on Effector Functions of Clone JT9, $CT8_{III}$, and $CT4_{II}$

Antiserum	Effector clone:	Percent specific lysis: JT9			$CT8_{III}$	$CT4_{II}$
	Target:	K562	Molt-4	Laz 221	Laz 156	Laz 156
Medium		81*	75	66	72	38
w6/32‡		89	81	79	22	39
Anti-p29/34§		90	73	70	66	3

* Standard deviation, <15%.
‡ Monoclonal antibody specific for HLA (class I).
§ Rabbit heteroantisera specific for human Ia antigens (class II).

complex as well as specific CTL to interact with target cells. However, JT9 and JT10 clones were selected after nonspecific mitogen stimulation and display a broad reactivity against genetically unrelated tumor cells. Since both JT9 and JT10 also express T3, T8, and T11 antigens, they consequently appear to be an additional population of CTL clearly distinct from the previously characterized alloreactive (or self-modified reactive) CTL. To substantiate this point, experiments were conducted to investigate whether JT9 cells would, like CTL, interact with targets via recognition of either class I or II products of the MHC. For this purpose, three representative target cell lines, K562, Molt-4, and Laz 221 were used in the experiments. K562 cells do not express either class I or class II antigens whereas Molt-4 cells express class I but not class II antigens. In contrast, both Ia and HLA structures are present on Laz 221 cells. $CT8_{III}$ and $CT4_{II}$, which are well-characterized human CTL clones directed at Laz 156 EBV-transformed B cells, were used as controls in these assays (19). As shown in Table V, it was found that preincubation of the three NK target cells with anti-HLA or anti-Ia antibodies did not affect subsequent lysis by JT9 cells. In contrast, as previously reported, preincubation of Laz 156 cells with anti-class I antibody resulted in a marked decrease of cytotoxicity mediated by $CT8_{III}$. Similarly,

preincubation of Laz 156 cells with anti-class II antibody was only effective in inhibiting cytotoxicity mediated by $CT4_{II}$ cells.

Discussion

A series of monoclonal antibodies termed anti-Ti have recently been produced using antigen-specific CTL clones for immunization. The target structures of these reagents were identified as clonally restricted 90 kD heterodimers. The anti-Ti antibodies were shown to block antigen-specific proliferation of CTL clones as well as cytotoxicity against antigen-bearing target cells. These functional effects strongly suggested that 90 kD clonotypic determinants represent antigen-receptor structures on specific T lymphocytes (15, 23).

In the present studies, mice were immunized with JT9 cloned cells, which represent NK active lymphocytes expressing a mature T cell phenotype ($T1^+$ $T3^+$ $T8^+$ $T11^+$ $T12^+$). These experiments were conducted to obtain monoclonal antibodies directed at cell surface structures involved in cytotoxic reactions performed by JT9 cells. One monoclonal antibody, termed anti-NKTa, was selected because of its capacity to block the cytotoxicity of JT9 towards K562 cells. Immunoprecipitation procedures indicated that anti-NKTa was directed at a heterodimer of identical 90 kD molecular mass as the anti-Ti antibodies. In addition, anti-NKTa was shown to have functional effects that are strikingly similar to those produced by anti-Ti. Namely, both anti-NKTa and anti-Ti block cytotoxic function and increase proliferative response to IL-2 of respective clones. Moreover, NKTa and T3 structures were found to co-modulate after interaction with corresponding antibodies. Such a linkage to the T3 molecule has been shown to represent a major characteristic of the Ti determinants (15, 23).

The identification of a 90 kD heterodimer on JT9 and JT10 cells represents a major step in the characterization of these clones. It indicates that NK active mature T lymphocytes are very closely related to specific CTL that have previously been shown to use a 90 kD-T3 molecular complex to interact with specific targets (15, 23). Nevertheless, JT9 and JT10 cells are representative of a population of T lymphocytes that is clearly distinct from previously characterized alloreactive or self-modified reactive cytotoxic T cells. NK active clones such as JT9 and JT10 were obtained after nonspecific mitogen stimulation (8, 14) and can kill a variety of unrelated target cells. Perhaps more importantly, JT9 and JT10 cells are cytotoxic for cells such as K562, that do not express HLA antigens, or Molt-4, that do not express Ia antigens. In addition, their cytotoxicity is not blocked by preincubation of representative target cells with either anti-class I or anti-class II antibodies. These findings are distinct from those obtained with the great majority of alloreactive CTL clones. Indeed, in the latter case, it has been found that $T4^+$ alloreactive CTL specifically interact with targets via recognition of class II antigens (19, 24) and that $T8^+$ alloreactive CTL interact with targets via recognition of class I antigens (19). The absence of genetic restriction for class I or class II products of the MHC complex appears to be a unique functional characteristic of mature T lymphocytes that have NK activity.

Based upon the fact that cytotoxicity of the great majority of $T8^+$ alloreactive CTL could be blocked by either anti-HLA antibodies at the target cell level or anti-T8 antibodies at the effector cell level, it has been postulated that the T8

molecule represents an associative recognition structure for a monomorphic region of class I antigens (25). In this context, the absence of blocking effects of anti-T8 antibodies on the cytotoxicity of JT9 cells correlates well with the absence of genetic restriction of these cells for class I antigens.

In contrast to anti-Ti antibodies available thus far, anti-NKTa antibody was found to react with two clones (JT9, JT10) and not only with the immunizing cells (JT9). Given the fact that individual NK clones can have different target specificities (14), it was of great interest to compare the specificity of JT9 and JT10 cells that express the identical 90 kD clonotype. It was found that JT9 and JT10 cells had a superimposable pattern of reactivity towards 15 different target cells tested. Although these results do not constitute a direct demonstration, they suggest that the NKTa structure is responsible for the target specificity of JT9 and JT10 NK active cells and is therefore functionally analogous to the Ti determinants present on alloreactive T cell clones. In addition, it is important to point out that NKTa-positive clones did not react with anti-Ti antibodies (data not shown) and, conversely, Ti-bearing clones did not react with anti-NKTa. This suggests that the NKTa determinant represents a variable region of the 90 kD heterodimer. Furthermore, two individual lines, JT9 and JT10, out of a relatively small number of NK clones tested, were found to express NKTa whereas only 1 out of 80 CTL clones directed against the same EBV-transformed B cell line, Laz 156, reacted with anti-Ti_{1A} and anti-Ti_{1B} (15). Although additional studies need to be performed to substantiate this point, these results suggest a much lower degree of diversity within the NK effector population than that found for alloreactive cytotoxic T cells.

Taken together, the data presented here leads to a working hypothesis concerning the nature of one population of "NK-active" lymphocytes represented by JT9 and JT10 cells. These cloned cells are mature T lymphocytes because they express the T3 structure that is involved in expression of their cytotoxic function. Although the NK active clones display a broad reactivity, they do have target specificity since, for example, they do not spontaneously kill EBV-transformed B cell lines, whereas strong cytotoxicity against these targets is obtained after lectin approximation (unpublished data). Furthermore, they express unique 90 kD heterodimeric determinants that are functionally linked to T3 antigen and which may represent the structural basis for their specificity.

The present studies support the view that at least one population of lymphocytes that mediate NK or NK-like activity may, in fact, represent an additional subset of mature "specific" T cells. The target antigen of these specific T cells is unknown at the present time. However, given the broad cellular reactivity of the NK clones, this antigen must have a relatively wide but yet restricted distribution on in vitro established cell lines commonly used in NK assays. In addition, this antigen does not appear to be recognized in association with either class I or class II products of the MHC complex. Identification of the target antigen of JT9 and JT10 cells will, of course, represent one of the goals for further studies. Perhaps more importantly, the nature of the other populations of human NK cells will be investigated in light of the present results. For this purpose, clones such as JT3 (14) that express T11 but not T3 structures or clones such as JT1 (7, 14) that do not express any presently identified T cell-associated molecules,

represent valuable tools. In future studies, it will be necessary to determine whether the specificity of these other types of NK clones is also associated with 90 kD structures or with different surface molecules. Hopefully, this approach will lead to a better understanding of the diversity of NK cells and ultimately help to clarify their biological significance.

Summary

The present studies were carried out to identify surface molecules involved in the cytotoxic effector function of a human natural killer (NK) clone termed JT9. This clone represents a mature T lymphocyte ($T3^{+}T8^{+}T11^{+}$) mediating NK-like activity. Using JT9 for immunization, one monoclonal antibody termed anti-NKTa was selected that blocked the cytotoxicity of the clone towards K562 cells. Reactivity of anti-NKTa antibody was assessed using a large panel of lymphoid and nonlymphoid cells including a variety of cloned cell lines with either cytotoxic T lymphocyte (CTL) or NK-like activity. Among all cells tested, only two individual clones, JT9 and JT10, were found to express NKTa antigen. JT10 was derived independently from the same individual as JT9 and also represents a mature T cell ($T3^{+}T8^{+}T11^{+}$) mediating NK-like activity. Like the Ti structure on CTL clones, the molecule defined by anti-NKTa was shown to be membrane associated with T3 in co-modulation experiments. Moreover, anti-NKTa precipitated a 90 kD heterodimeric structure in sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of ^{125}I surface-labeled JT9 cells.

The blocking capacity of anti-NKTa was evaluated in cytotoxicity assays using a panel of target cells. The influence of anti-T3 was tested in parallel and it was found that both anti-NKTa and anti-T3 blocked the cytotoxicity of the cloned cells against all targets. Given the potential role of 90 kD molecules as antigen-receptor structures, the specificity of the two $NKTa^{+}$ NK clones was compared and found superimposable when assessed using 15 in vitro established cell lines. However, in contrast to conventional CTL clones, the expression of cytotoxicity by JT9 and JT10 was not dependent upon recognition of class I or class II major histocompatibility complex gene products on the target cells. In addition, the cytotoxicity of these $T8^{+}$ NK active clones could not be blocked by anti-T8 antibodies. Taken together, the present data suggest that the specificity of one population of human NK active lymphocytes is determined by clonotypic structures. The NKTa determinant identified here appears to belong to the same family of molecules as Ti structures, previously identified on antigen-specific T lymphocytes.

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