γ/δ T CELL CLONES AND NATURAL KILLER CELL CLONES MEDIATE DISTINCT PATTERNS OF NON-MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED CYTOLYSIS

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Most T cells express the antigen-specific MHC-restricted TCR-α/β, whereas the TCR-γ/δ is expressed on small subpopulations of PBL and thymocytes (1, 2). Cell-mediated lysis of certain tumor targets without restriction by the MHC can be accomplished by NK cells (3–5) and γ/δ T cells (6–9). Despite intensive studies, the mechanism of non-MHC-restricted E/T cell recognition remains unclear (10, 11). In general, distinct patterns of non-MHC-restricted cytolysis have not been associated with particular clones of effector cells and their surface structures.

To study the specificity and function of T cells bearing the TCR-γ/δ (11–14), we compared the non-MHC-restricted cytotoxic activity of γ/δ and NK cell clones. We examined NK clones and γ/δ T cell clones derived from the same healthy individuals for target selectivity, and found that the γ/δ clones mediated a pattern of cytotoxicity on tumor cell lines that was markedly different from that mediated by NK cell clones. The cytotoxic activity of the γ/δ T cell clones was influenced by mAbs directed against the CD3/TCR-γ/δ complex. Our findings indicate that the TCR-γ/δ may be involved in the distinct pattern of non-MHC-restricted cytolysis mediated by γ/δ T cell clones on certain tumor target cells such as the Daudi cell line, possibly at the level of E/T cell recognition.

Materials and Methods

Cytotoxic Clones. PBMC were isolated by Ficoll-Hypaque sedimentation of heparinized blood samples; most experiments described involve PBL obtained from one healthy volunteer blood donor. These were positively selected by FACS for cells expressing the Leu-19 antigen (predominantly NK cells), the CD16 (Leu-11a) antigen (NK cells), or γ/δ T cells. The sorted cells were cloned by limiting dilution at 0.3 cells per well (5, 6, 15). The culture medium

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[1] *Abbreviations used in this paper:* CML, cell-mediated lysis; LCL, lymphoblastoid B cell lines.

was RPMI 1640, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all from Whittaker M. A. Bioproducts, Walkersville, MD), 25 mM Hepes buffer (Sigma Chemical Co., St. Louis, MO), and 10% heat inactivated human serum (Pel-Freeze Biologicals, Rogers, AR). The plated cells were stimulated with 500 U/ml rIL-2 (Hoffmann-La Roche, Nutley, NJ) and a mixture of irradiated allogeneic or autologous feeder cells (2×10^4 PBL and 10^4 EBV-transformed lymphoblastoid B cell lines [LCL]) as described (5, 6). The clones were expanded by replating at weekly intervals with feeder cells, fresh medium, IL-2, and PHA (0.25 μg/ml; Wellcome Diagnostics, Beckenham, UK).

mAbs and Immunofluorescence. For immunofluorescence studies, cells were labeled with mAbs reactive against CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2b), CD16 (Leu-11a), CD56 (Leu-19) epitopes, and TCR-α/β (WT31) (all from Becton Dickinson & Co., Mountain View, CA). mAbs reactive with all cells expressing the TCR-γ/δ were TCR-δ1 (T Cell Sciences, Cambridge, MA) and TCR-γ/δ-1 (8, 16). In addition, some γ/δ clones were stained with mAbs that detect specific V region determinants on the TCR-γ/δ. These mAbs were TiγA (a gift from F. Triebel and T. Hercend [17]), BB3 (a gift from L. Moretta [18]), and δ TCS1 (provided by T Cell Sciences [16]). mAbs against LFA-1α (TS1/22), LFA-1β (TS1/18), ICAM-1 (RR1/1), and LFA-3 (TS2/9) were donated by T. Springer (Center for Blood Research, Boston, MA). For direct fluorescence, cells were labeled with mAbs conjugated to FITC or phycoerythrin (PE) in PBS (30 min at 4°C) and washed. For indirect fluorescence, cells were exposed to mAbs, subsequently incubated with FITC-conjugated goat anti-mouse antibody (Becton Dickinson & Co.), and washed before analysis by FACS. In each experiment, nonspecific binding was subtracted using appropriate isotype controls. The mAbs included in cytotoxicity assays were TCR-γ/δ-1; mAb reactive to the $T11_2$ and $T11_3$ epitopes of the CD2 molecule (a gift from S. Schlossman, Dana-Farber Cancer Institute, Boston, MA), mAb reactive to CD3 (UCHT1, kindly provided by P. Beverley, University College Hospital, London, UK), and mAb directed against HLA-DR (L234), -DQ (S3/4), and -DP (B7/21.2), (donated by F. Bach, University of Minnesota, Minneapolis, MN).

Cell-mediated Lysis (CML) Assay. The clones were washed and assayed for cytotoxicity by incubation in 96-well round-bottomed microtiter plates (Costar, Cambridge, MA) with 5×10^3 ^{51}Cr-labeled target cells for 4 or 6 h at 37°C as described (19). Percent specific lysis was calculated as 100× [(counts per minute released with effectors − counts per minute released alone)/(counts per minute released by detergent) − (counts per minute released alone)]. Cytotoxicity values obtained from serial E/T ratios were converted into LU. One LU was defined as the number of effector cells required to achieve 20% specific lysis of 5×10^3 targets. Tumor target cell lines (19) K562, Molt 4, U937, and Raji were obtained through the American Type Culture Collection (Rockville, MD). The Daudi cell line and the variant β_2m-Daudi (transfected with the mouse β_2-microglobulin gene and thus expressing MHC class I antigens) were a gift from J. Parnes (Stanford, CA). The LCL TK6 is a derivative of WI-L2, obtained from W. Thilly (M. I. T., Cambridge, MA). These cell lines were maintained in RPMI containing 10% FCS (HyClone Laboratories, Logan, UT). Mycoplasma contamination was excluded.

Results and Discussion

Initially, we examined distinct tumor target cell lines (15, 19) for cytolysis by these clones (Table I). Clones selected by FACS for TCR-γ/δ expression from a culture primed to the LCL TK6 mediated a very high level of cytotoxicity on Daudi cells but failed to lyse the Raji cell line (Table I). In marked contrast, NK clones obtained from PBL of the same donor lysed the Daudi and Raji targets equally well (Table II). Disparate lysis of these two target lines by γ/δ clones and NK clones was remarkable since both Daudi and Raji are quite similar cells derived from Burkitt's lymphomas. The Daudi cell line is deficient for HLA class I antigens due to defective β_2m synthesis but expresses HLA class II antigens, while Raji expresses both HLA class I and II. Although non-MHC-restricted lysis appears to correlate inversely with the expression of HLA class I antigens on some target cells (20, 21), this does

TABLE I

Clones Obtained by Sorting for TCR-γ/δ-1⁺ Cells from Primed Culture

Clone	Type	Percent specific binding of mAb						Percent cytotoxicity			
		CD3	TCR-δ1	CD16	CD56	CD4	CD8	K562	Daudi	Raji	TK6
GPC4	γ/δ	99	100	0	39	0	95	42	53	2	12
GPE5	γ/δ	99	99	0	30	0	1	27	66	0	25
GAK4	γ/δ	99	99	0	30	0	43	41	58	3	35
GAC1	γ/δ	100	99	9	63	0	1	15	59	4	18
GAH1	γ/δ	99	99	2	3	ND	ND	38	56	0	25
GAH2	γ/δ	100	99	0	63	0	15	58	78	6	22
GAF2	γ/δ	100	100	ND	56	0	86	34	61	5	12
GAG1	γ/δ	99	97	ND	79	0	55	58	78	5	22
GAG2	γ/δ	99	99	ND	76	0	1	42	57	2	13
GAB1	NK	0	0	0	99	0	0	85	79	100	89

The clones were derived from a culture of PBL primed to the LCL TK6 for 7 d followed by FACS sorting for TCR-γ/δ⁺ cells. Clones were screened for cytotoxicity on the indicated target cell lines. The results indicating percent cytotoxicity were obtained in experiments where equal volumes (25 μl) of medium in wells containing the clones were transferred to plates to which the target cells were subsequently added. Two or three replicates were performed per clone; SD for percent cytotoxicity was usually <3%. Autologous γ/δ clones and NK clones were simultaneously compared. The experiments in Tables I, II, IV, and VI represent 2 of 24 similar experiments. The phenotypes of the clones in Table I were determined by direct immunofluorescence. Values shown for binding of mAbs are expressed as percent positive cells, and data are rounded to the decimal point. Clone type designations for Tables I–V are defined as NK for CD3⁻ cells that are CD16⁺ and/or CD56⁺; γ/δ for CD3⁺ cells that were found to be TCR-γ/δ-1 or TCR-δ1⁺ and WT31⁻; and α/β for CD3⁺ cells that are TCR-γ/δ-1⁻ and WT31⁺. The results shown reflect typical response patterns selected from 284 clones evaluated for Table I.

not provide an explanation for the particular pattern of cytotoxicity by these γ/δ clones. First, virtually all NK clones lysed Raji as well as or even better than Daudi targets and, second, an HLA class I-expressing Daudi variant was strongly killed by all the γ/δ clones (Table III). All γ/δ clones and NK clones mediated non-MHC-restricted cytolysis against HLA antigen-deficient K562 target cells. Both NK and

TABLE II

Clones Obtained by Sorting for CD16⁺ Cells from PBL

Clone	Type	Percent specific binding of mAb				Percent cytotoxicity			
		CD3	TCRδ1	CD16	CD56	K562	Daudi	Raji	TK6
NAD1	NK	2	0	94	99	82	54	47	52
NAA1	NK	1	0	91	97	83	81	86	23
NAA3	NK	1	ND	93	99	73	75	85	59
NAC2	NK	3	ND	99	99	61	11	53	31
NEE6	NK	2	ND	98	96	86	41	46	25
NEE1	NK	1	ND	99	99	90	55	60	54

The clones were derived from fresh PBL sorted for CD16 (Leu-11)⁺ cells. LCL used as stimulators were TK6. Clones were screened for cytotoxicity on the indicated target cell lines. The results indicating percent cytotoxicity were obtained in experiments where equal volumes (25 μl) of medium in wells containing the clones were transferred to plates to which the target cells were subsequently added. Two or three replicates were performed per clone; SD for cytotoxicity was usually <3%. Autologous γ/δ clones and NK clones were simultaneously compared. The phenotypes of the clones in Table II were determined by direct immunofluorescence. Values shown for binding of mAbs are expressed as percent positive cells, and data are rounded to the decimal point. Clone type designations are defined as in Table I. The results shown reflect typical response patterns selected from 90 clones evaluated for Table II.

TABLE III

Target Selectivity of Representative Clones

				Percent cytotoxicity on target cell line					
Clone	LCL	Type	E/T	K562	Daudi	β_2m Daudi	Molt 4	Raji	TK6
RAD6	TK6	NK	9	80	81	71	86	82	68
			3	72	62	56	77	78	57
			1	58	31	28	58	41	40
GAB1	TK6	NK	3	70	70	65	57	81	68
			1	75	57	52	41	67	57
			0.3	64	28	20	25	43	41
BC5	TK6	NK	9	73	60	68	60	81	35
			3	49	33	38	47	54	17
			1	26	19	15	24	32	7
GPC4	TK6	γ/δ	6	44	79	69	50	1	28
			2	32	85	63	47	1	23
			0.7	25	83	57	28	2	16
GAC1	TK6	γ/δ	9	14	70	63	27	6	11
			3	6	63	61	18	4	9
			1	2	42	40	9	2	7
FB5	PF	γ/δ	9	53	77	74	70	6	42
			3	43	74	62	58	3	33
			1	23	72	65	41	1	31
MH3	RM	γ/δ	9	11	61	ND	40	3	42
			3	9	52	ND	40	3	35
			1	3	49	ND	35	1	29
PI4	PS	γ/δ	9	62	81	71	86	9	34
			3	31	72	63	63	7	13
			1	16	73	59	50	1	5
AP1	TK6	α/β	9	51	5	8	29	9	41
			3	28	2	2	5	5	17
			1	14	1	0	1	1	5
AW4	PF	α/β	9	46	0	1	56	4	45
			3	22	2	1	43	2	23
			1	7	9	9	25	1	5

Percent cytotoxicity mediated by 10 representative clones is shown at three E/T ratios when tested for lysis against the six target cell lines indicated. The clone type designations are listed as in Table I. LCL indicates the particular stimulator LCL for each of these clones.

γ/δ T cell clones also lysed the cell lines TK6, Molt4 (Tables I-V), and U937 (not shown). However, in contrast to NK clones, lysis of these target cells was weaker than the cytotoxicity against Daudi cells if lower E/T ratios were compared (Table III).

This characteristic cytotoxic pattern was independent of priming to the LCL TK6 in vitro before FACS selection. Clones obtained after sorting of fresh PBL for TCR-γ/δ-1$^+$ cells and stimulation with different feeder LCL (including the autologous LCL) had an identical pattern of cytotoxicity (Table IV). All 356 γ/δ clones that were obtained from this donor's fresh PBL without "priming" to TK6 mediated high levels of lysis on Daudi cells but virtually no killing on the Raji cell line. These clones

TABLE IV
Clones Obtained by Sorting for TCR-γ/δ-1⁺ Cells from PBL

Clone	Type	Percent specific binding of mAb					Percent cytotoxicity				
		CD3	WT31	TCR-γ/δ-1	CD4	CD8	K562	Daudi	Raji	TK6	LCL
FB5	γ/δ	100	3	100	8	6	66	86	29	56	0
FK4	γ/δ	100	2	100	1	2	61	67	8	47	3
MB5	γ/δ	100	0	100	2	1	50	68	11	21	4
MB6	γ/δ	100	2	99	0	1	73	61	4	18	4
MC1	γ/δ	99	2	99	3	5	55	60	7	12	3
ME1	γ/δ	99	ND	99	0	90	69	74	12	30	0
MH3	γ/δ	98	5	100	ND	ND	39	80	4	40	1
MK4	γ/δ	100	ND	100	0	24	70	70	9	23	10
PC1	γ/δ	100	3	100	1	2	72	80	9	0	0
PD4	γ/δ	99	1	100	1	1	65	71	6	23	6
PI4	γ/δ	99	5	100	1	4	64	72	6	22	0
PJ2	γ/δ	100	1	99	0	1	49	67	2	6	0

The clones were derived from fresh PBL sorted for TCR-γ/δ-1⁺ cells. LCL used as stimulator cells were PF-, PS-, and RM-LCL for clones with initials F, P, and M, respectively. Clones were screened for cytotoxicity on the indicated target cell lines including the specific stimulator LCL. The results indicate percent cytotoxicity were obtained in experiments where equal volumes (25 μl) of medium in wells containing the clones were transferred to plates to which the target cells were subsequently added. Two or three replicates were performed per clone; SD for percent cytotoxicity was usually <3%. Autologous γ/δ clones and NK clones were simultaneously compared. The phenotypes of the clones in Table IV were determined by indirect immunofluorescence. Values shown for binding of mAbs are expressed as percent positive cells, and data are rounded to the decimal point. Clone type designations are defined as in Table I. The results shown reflect typical response patterns selected from 211 clones evaluated for Table IV.

also killed K562, and many but not all clones also effectively destroyed TK6. However, these γ/δ clones did not lyse the particular LCL used to stimulate them, except for TK6. These stimulator LCL were susceptible to cytolysis by NK clones and alloreactive α/β T cell clones. The exclusive pattern of cytotoxicity by these γ/δ T cell clones was reproduced in >40 experiments with some of these clones. Moreover, analogous results were obtained when these same γ/δ clones (seven of seven) were tested in Rotterdam on TK6 cells and on independently maintained K562, Daudi, Raji, and LCL targets. In contrast to these γ/δ clones, all TCR-α/β clones with non-MHC-restricted cytotoxic activity against K562 that were obtained from the same donor (33 clones) did not lyse Daudi or Raji cells, although many clones lysed Molt4 and/or TK6 targets (representative clones shown in Tables III and IV).

Phenotypic analysis with mAbs (Tables I, II, IV, and V, Figure 1) identified the NK clones as Leu-11a (CD16)⁺ or Leu-11a⁻ and Leu-19 (CD56)⁺, but TCR-δ1⁻ while the γ/δ clones were TCR-γ/δ-1⁺, TCR-δ1⁺ (mAbs reactive with all γ/δ cells [8, 16]). Most γ/δ T cell clones were double negative for CD4 and CD8, but some clones expressed variable levels of CD8. Particularly striking were rare clones that markedly lysed Raji, obtained from cells that had been sorted for TCR-γ/δ-1⁺. All of these were subsequently proven to be NK clones rather than γ/δ T cell clones (presumably contaminants in the sorted cells, i.e., clone GAB1; Table I, Fig. 1). Furthermore, some clones that were derived from a sort for Leu-19⁺ PBL mediated the pattern of lysis corresponding to that of the γ/δ clones (Table V). Indeed, all such clones were shown to be Leu-19⁺ γ/δ T cell clones and not NK clones; clones that displayed

TABLE V

Clones Obtained by Sorting for CD56⁺ Cells from PBL

Clone	Type	Percent specific binding of mAb					Percent cytotoxicity			
		CD3	WT31	TCRγδ-1	CD16	CD56	K562	Daudi	Raji	TK6
RAD6	NK	0	0	0	99	99	70	64	88	57
CE1	NK	0	0	0	100	100	79	9	7	1
BD2	NK	2	2	2	97	100	80	69	80	61
BC5	NK	2	1	2	90	95	30	43	50	14
AD1	NK	3	3	4	90	99	43	18	30	0
BC7	NK	2	ND	ND	90	99	74	81	84	88
AF1	NK	1	0	1	87	98	84	84	84	91
BG5	NK	2	ND	1	70	99	87	91	82	18
BE4	NK	2	ND	ND	66	100	79	77	67	92
BD6	NK	4	ND	ND	29	97	77	83	79	64
BB10	NK	1	0	0	18	84	54	37	40	85
BG14	α/β	100	100	1	8	59	40	1	1	60
AG2	α/β	99	100	0	2	0	0	0	1	1
AP1	α/β	100	100	5	1	0	47	0	1	32
BB6	γ/δ	92	3	88	6	62	76	78	17	10
BB1	γ/δ	98	9	95	25	59	37	35	4	1
CC5	γ/δ	100	3	97	3	9	68	82	6	45
BG3	γ/δ	98	5	98	7	100	45	56	7	6
BC3	γ/δ	99	12	98	9	53	76	78	16	92
AM4	γ/δ	99	3	98	1	49	63	75	8	51

The clones were derived from fresh PBL sorted for Leu-19 $(CD56)^+$ cells. LCL used as stimulator cells were TK6. Clones were screened for cytotoxicity on the indicated target cell lines. The results indicating percent cytotoxicity were obtained in experiments where equal volumes (25 μl) of medium in wells containing the clones were transferred to plates to which the target cells were subsequently added. Two or three replicates were performed per clone; SD for percent cytotoxicity was usually <3%. Autologous γ/δ clones and NK-clones were simultaneously compared in each experiment. The phenotypes of the clones in Table V were determined by indirect immunofluorescence. Values shown for binding of mAbs are expressed as percent positive cells, and data are rounded to the decimal point. Clone type designations are defined as in Table I. The results shown reflect typical response patterns selected from 338 clones evaluated for Table V.

a different pattern of cytotoxicity were proven to be NK clones or, rarely, TCR α/β clones. 20 representative γ/δ clones were phenotyped with additional mAbs that react with specific V region-encoded protein products and thus detect functional TCR gene rearrangements. As described for the majority of peripheral blood γ/δ cells (16), all 20 clones were $TiγA^+$ (reactive with Vγ9 [17]) and $BB3^+$ (reactive with Vδ2 [18]). However, all these clones lacked the determinant recognized by mAb δTCS1 (reactive with Vδ1[16]). The phenotype of these clones is consistent with the FACS analysis of this donor's peripheral blood γ/δ cells that seem $BB3^+$ and $TiγA^+$, but are $δTCS1^-$.

Since the expression of the TCR-γ/δ correlated with the particular pattern of cytotoxicity on these tumor cell lines, we investigated the role of the CD3/TCR-γ/δ complex in target cell lysis (Figs. 2 and 3). The effect of anti-CD3 mAb depended on FcR expression on target cells (5, 9, 22). The antibody was inhibitory when included in the cytotoxicity assay with FcR^- targets such as Molt 4 and TK6 (not shown), while the lysis of the FcR^+ Daudi and Raji cells (Fig. 2) was enhanced due to cross-linking of the effector and target cells via FcR (9, 22). Induction of Raji cells lysis

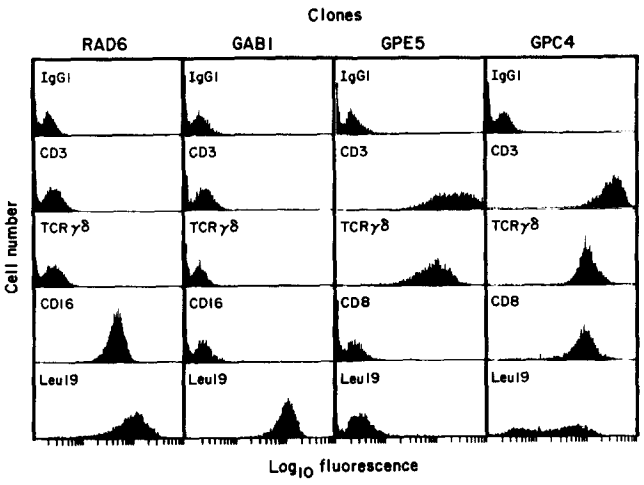

FIGURE 1. Phenotype of selected NK clones and γ/δ T cell clones. Cloned cells (representative from Tables I–III) were stained with fluorescent mAbs for direct immunofluorescence. 5,000 cells were analyzed by FACS for each mAb. The NK clones are designated RAD6 ($CD16^+$, Leu-19^+, $CD2^+$, $CD3^-$, TCR-$\delta1^-$) and GAB1 ($CD16^-$, Leu-19^+, $CD2^+$, $CD3^-$, TCR-$\delta1^-$); the γ/δ clones are GPE5 ($CD2^+$, $CD3^+$, TCR-$\delta1^+$, $\delta TCS1^-$, $BB3^+$, $Ti\gamma A^+$, $CD4^-$, $CD8^-$, $CD16^-$, Leu-19^{weak}) and GPC4 ($CD2^+$, $CD3^+$, TCR-$\delta1^+$, $\delta TCS1^-$, $BB3^+$, $Ti\gamma A^+$, $CD4^-$, $CD8^+$, $CD16^-$, Leu-19^+) with the additional markers not shown.

by anti-CD3 mAb indicates that the very inefficient lysis of Raji cells by effector γ/δ T lymphocytes in the absence of anti-CD3 was due to nonrecognition rather than intrinsic resistance of Raji cells to the lytic machinery of these clones. Appropriate concentrations of the TCR-γ/δ-1 mAb (which is of the same isotype as the anti-CD3 mAb) were inhibitory for the γ/δ T cell–mediated lysis of Daudi, Molt 4, and, although less, for TK6 target cells (Figs. 2 and 3). On the contrary and in agreement with published data (9, 23), the lysis of FcR^+ K562 and U937 target cells was augmented in the presence of TCR-γ/δ-1 mAb. Nevertheless, in some experiments, higher concentrations of the TCR-γ/δ-1 mAb had a mild inhibitory effect on the lysis of K562 (as shown in Fig. 3) and U937 cells (not shown). This effect may result from a competition between stimulatory and inhibitory signals depending on the mAb, its concentration, and the mechanism of E/T cell interaction. The stimulatory signals may include in this system FcR mediated crosslinking (9, 22) and the augmentation of E/T adhesion through LFA-1 after CD3 crosslinking (24). The inhibitory signals may be a consequence of the interference of the mAb with specific recognition structures on the effector cell and/or an intracellular negative signal affecting the efficacy of T cell stimulation (8, 25). As the concentration of the TCR-γ/δ-1 antibody increased, the inhibitory effects outweighed the inducing capacity on FcR^+ targets and, thus, the overall results shifted towards inhibition. In contrast to some reported findings (26, 27) showing that only very low mAb concentrations were blocking the cytotoxic activity by γ/δ T cells against allogeneic lymphoblastoid cell lines (favoring the idea of transmission of a negative signal), we detected maximal inhibition at high concentrations. This is compatible with the view that the mAb interfered with the TCR-mediated recognition and/or delivered negative signals. Incubation of the clones for 18 h in culture medium containing either the anti-CD3 or TCR-γ/δ-1 mAb caused a marked decrease of TCR density on these γ/δ cells (not shown). Preincubation of the clones with the anti-CD3 mAb (followed by washing) augmented lysis of the Daudi and Raji cells but inhibited lysis of Molt 4, and their pretreatment with the TCR-γ/δ-1 mAb abrogated the cytolysis on both Daudi and

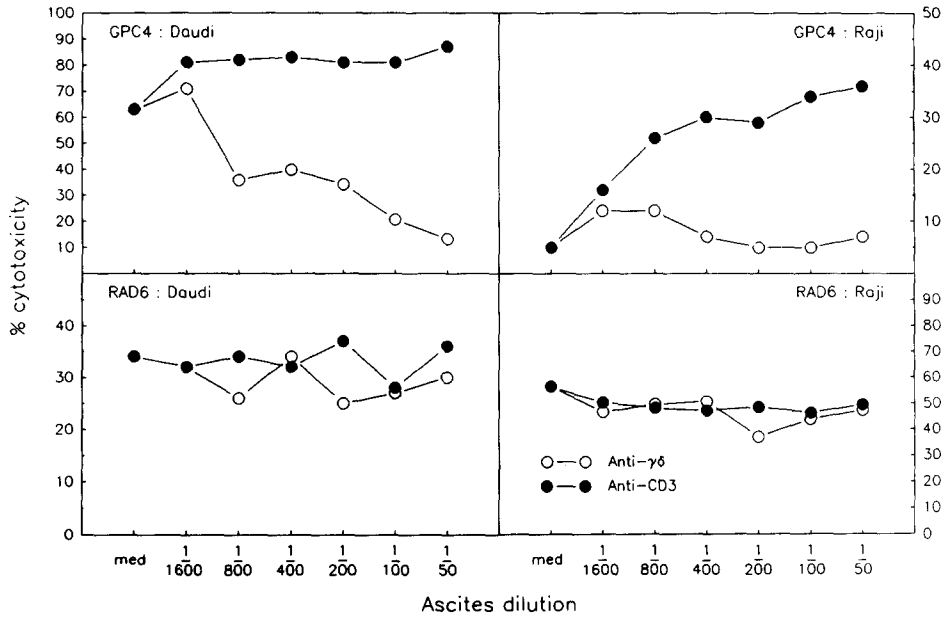

FIGURE 2. Specific effect of anti-CD3 and TCR-γ/δ-1 mAbs on cytolysis by BB3/TiγA$^+$ γ/δ T cell clones. Representative γ/δ T cell clone GPC4 (TCR-γ/δ-1$^+$, CD3$^+$) and NK clone RAD6 (TCR-γ/δ-1$^-$, CD3$^-$, CD16$^+$, Leu-19$^+$) were tested (4-h ^{51}Cr release assay) for the lysis of Daudi and Raji cells (E/T ratio of 3:1 shown). Cytotoxicity was measured in medium (med) and in medium containing the indicated dilution (vol/vol; indicated on the abscissa) of mouse ascites with anti-CD3 mAb (UCHT1, IgG1) or purified TCR-γ/δ-1 mAb (IgG1). Both mAbs were present throughout the assay. Other γ/δ clones were also studied (GPE5, MB5, MH3, FB5, and PI4) and showed similar results.

Molt4 targets (Fig. 4). Although most of the TCR had been downmodulated by TCR-γ/δ-1 or anti-CD3 pretreatment, a further exposure to anti-CD2 or anti-CD3 mAb, added in the beginning of the cytotoxicity assay, still resulted in strong lysis of both Raji and Daudi targets (Fig. 4). Thus, the blocking of cytotoxicity by TCR-γ/δ-1 mAb was caused by neither a simple inhibition of the effectors' lytic machinery nor toxicity to the cell. The cytotoxic activity against Daudi cells, but not Raji cells, was displayed by all BB3/TiγA$^+$ γ/δ clones. This lytic pattern contrasts with the distinct target cell susceptibility found in NK clones and α/β T cell clones. Moreover, lysis of some target cells can be blocked by TCR-γ/δ-1 mAb while the lytic machinery of these cells appears intact. These results suggest that the CD3/TCR-γ/δ complex on our γ/δ T cell clones could be involved in the recognition of determinants on certain targets such as Daudi cells. The lysis of K562 and U937 cells by our γ/δ clones was not effectively blocked in the presence of anti-TCR mAb. It is difficult to determine if this apparent lack of anti-TCR blocking of lysis resulted from a deficit of inhibitory signals or a predominance of stimulatory signals. Relevant to this may be the observation that the capacity of all γ/δ and α/β clones to kill K562 and U937 disappeared when the clones were passaged in vitro for many weeks (although our γ/δ clones maintained the ability to lyse Daudi cells). These findings may suggest that the cytotoxic activity by our γ/δ clones against K562 and U937 cells was medi-

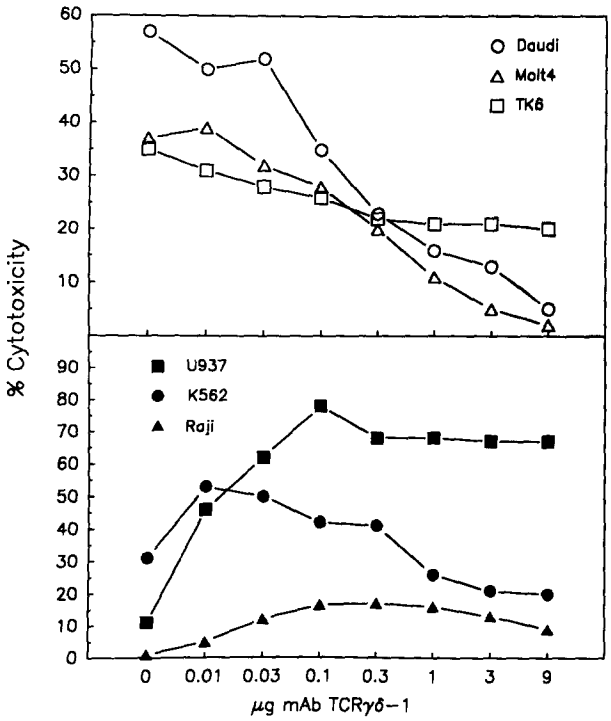

FIGURE 3. Influence of TCR-γ/δ-1 mAb on lysis by BB3/TiγA$^+$ γ/δ T cell clones on different target cells. The effects of TCR-γ/δ-1 mAb were studied on target cells for BB3/TiγA$^+$ clones (performed as the experiment shown in Fig. 2). Representative clone GPC4 (E/T ratio 10:1) was incubated with serial dilutions of the mAb and tested for lysis on the target cells indicated in a 5-h cytotoxicity assay. The results for this clone in one such experiment are shown for Daudi, Molt 4, and TK6 in the upper panel and for U937, K562, and Raji cells in the lower panel. Five other clones were studied with qualitatively similar results, although in some experiments, somewhat greater inhibition on lysis of TK6 was observed.

ated differently than that against Daudi. Our data corroborate recent results reporting a similar specificity of polyclonal BB3/TiγA$^+$, δTCS1$^-$ T cell lines (23) and the inhibitory effects of anti-TCR mAb on the lysis of K562, U937 (9, 23), and Daudi cells (23), as well as Molt 4 cells (9, 28). Other investigators (27) have reported that the lysis of Molt 4 cells by allospecific δTCS1$^+$ γ/δ T cell clones is augmented in the presence of anti-CD3 or δTCS1 mAb, and only Leu-19$^+$ displayed non-MHC-restricted activity. Their results are not necessarily in contrast to our data since our clones are clearly different: our clones are all δTCS1$^-$ and mediate non-MHC-restricted cytotoxicity regardless of Leu-19 expression.

An alternative explanation for blocking of cytotoxicity by anti-TCR mAb could be negative signaling elicited by certain types of TCR occupancy. Since the TCR-γ/δ may not be directly involved in the γ/δ T cell/target cell interaction, there is a possibility that the cell surface molecules mediating effector target cell adhesion (29), such as LFA-1, ICAM-1, CD2, and LFA-3, are the essential recognition structures. However, we have found these receptors equally expressed on all γ/δ T cell clones, NK$^-$, and α/β T cell clones examined, and we identified no ligand on Daudi (LFA-1$^-$, ICAM-1$^+$, CD2$^-$, LFA-3$^-$) that was not present on Raji (LFA-1$^+$, ICAM-1$^+$, CD2$^-$, LFA-3$^+$) (not shown). Therefore, it seems quite unlikely that the differential expression of these molecules could account for the particular pattern of cytolysis displayed by our γ/δ T cell clones. Nevertheless, other yet unknown structures distinct from the TCR and these adhesion molecules could be functional in the lytic mechanism used by γ/δ T cells.

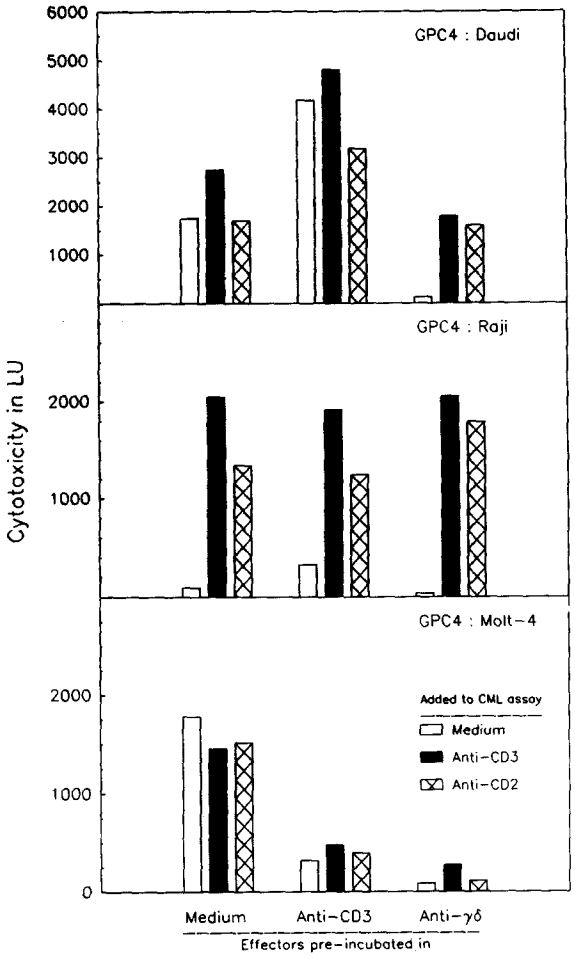

FIGURE 4. Triggering of lysis through CD3 and CD2 epitopes after modulation of the TCR-γ/δ. Clone GPC4 was incubated for 18 h in medium, or with either anti-CD3 mAb (UCHT1, 1:200) or TCR-γ/δ-1 (1:100). This resulted in marked, although not complete, modulation of the TCR-γ/δ and CD3 molecules (not shown). After washing (three times), cells were tested for lysis of Daudi, Raji (both FcR^+), and Molt 4 (FcR^-) (E/T ratios of 5, 1, and 0.2 to 1; 6-h CML assay). Data are expressed as LU per 10^6 effectors. Anti-CD3 mAb (UCHT1 at 1:200, vol/vol), or a mixture of anti-$T11_2$ plus anti-$T11_3$ mAb (1:800, vol/vol) were added to some of the wells. A similar pattern was seen with clone GPE5; NK clones incubated with anti-CD3 and TCR-γ/δ-1 mAbs in parallel were not affected in the lysis of these targets (not shown).

Despite the great potential diversity of the TCR-γ/δ (predominantly due to the junctional diversity of the δ chain) (1, 2, 30, 31), the number of functional gene combinations utilized by human peripheral γ/δ T cells appears quite limited (16–18). The cytotoxicity pattern by the BB3/TiγA$^+$ γ/δ clones was the same when using clones obtained from two additional blood donors and comparing them with NK and α/β clones derived from each of these donors (not shown). Furthermore, a similar target spectrum (lysis of Daudi but not the stimulator LCL) and the same productive Vγ/δ gene expression (assessed by mAb BB3 and TiγA) was independently detected in all TCR-γ/δ-1$^+$, δTCS1$^-$ clones derived from four different donors in Rotterdam. The results presented here strongly suggest that human BB3/TiγA$^+$ T cells recognize different determinants than non-MHC-restricted NK cells (3, 4) or IL-2-activated MHC-restricted α/β T cells (31). The relationship of these target structures to known antigens for γ/δ T cells remains to be determined (12–14, 32). However, these target structures cannot be related to HLA class I since Daudi cells do not express β_2m-associated determinants. The target molecules are probably not

typical HLA class II determinants since 25 allogeneic LCL including the stimulator LCL (except for TK6), which express a range of diverse class II antigens, were resistant to lysis by all our γ/δ clones (not shown). Furthermore, mAbs directed against HLA-DR, -DP, or -DQ did not inhibit lysis of Daudi by these γ/δ cells (not shown). It is noteworthy that all γ/δ clones that had been derived with autologous LCL and PBL stimulators, and thus were never exposed to allogeneic determinants in vitro, still mediated the typical pattern of cytolysis. These results support the idea that our γ/δ clones probably do not recognize polymorphic or nonpolymorphic MHC molecules as suggested for other γ/δ cells (26, 28, 33–35). It is conceivable that our γ/δ cells could recognize relatively nonpolymorphic determinants that are expressed in sufficient quantities on only a minority of target cells. If the TCR-γ/δ is in fact the receptor that reacts to such determinants, it is possible that such epitopes function as "superantigens" for BB3/TiγA$^+$ cells. Indeed, the epitopes on some tumor targets could be related to heat-shock proteins contained in mycobacterial preparations (12–14, 32, 36). It will be interesting to compare the function and specificity of our BB3/TiγA$^+$ clones with δTCS1$^+$ clones, other non-MHC-restricted cytolytic γ/δ T cells (26–28), and clones derived from different tissues (30, 32). Although the physiologic role and function of γ/δ T cells remains elusive, our data clearly indicate that the recognition of some target cells by our BB3/TiγA$^+$ T cell clones is markedly different from other non-MHC-restricted lymphoid cells.

Summary

Non-MHC-restricted killer cells are cytotoxic lymphocytes that can mediate cytolysis of most tumor targets without apparent selectivity and restriction by the MHC, particularly when activated with IL-2. These effector cells include predominantly NK cells and T cells expressing the TCR-γ/δ. We found that TCR-γ/δ-1$^+$, δTSC1$^-$, BB3$^+$, TiγA$^+$ T cell clones mediate a characteristic cytolytic pattern of non-MHC-restricted cytolysis that is markedly different from NK clones and α/β T cell clones derived from the peripheral blood of the same normal individuals. The characteristic finding is that all BB3/TiγA$^+$ γ/δ clones mediate strong cytolysis of Daudi cells but they do not lyse Raji cells. In contrast, NK clones from the same donors mediate strong cytolysis of both Daudi and Raji targets. Cytotoxicity by the γ/δ clones on certain target cells such as Daudi and Molt 4 can be specifically inhibited by mAbs reactive against the TCR-γ/δ. Therefore, the TCR-γ/δ on these clones either directly recognizes target epitopes on some tumor targets or it is involved in the regulation of their cytotoxic function. The expression of TCR-γ/δ products reacting with the BB3 and TiγA mAbs reflects the usage of identical TCR-γ/δ V region genes that appear to be associated with the characteristic pattern of non-MHC-restricted cytotoxicity displayed by this major subset of human peripheral blood γ/δ cells.

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