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HUMAN CYTOTOXIC LYMPHOCYTES

V. Frequency and Specificity of $\gamma\delta^+$ Cytotoxic Lymphocyte Precursors Activated by Allogeneic or Autologous Stimulator Cells[1]

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We have investigated the frequency and specificity of $\gamma\delta^+$ cytotoxic lymphocyte precursors (CLP) under limiting dilution culture conditions. E rosette separated total T cells and $CD3^+CD4^-CD8^-TCR\alpha\beta^-$ double-negative (DN) T cells were cocultured with allogeneic or autologous PBMC stimulator cells, and frequencies of alloreactive and autoreactive CLP were determined after 12 to 14 days against Con A blast target cells. Freshly isolated DN cells consisting of 82.3 ± 8.2% $\gamma\delta^+$ T cells did not exert cytolytic activity against K562 or anti-TCR $\gamma\delta$ mAb-producing hybridoma cells. In striking contrast to E^+ cells, the vast majority of alloantigen-stimulated clonally developing DN CLP did not show specificity for stimulator-derived target cells. Thus, frequencies of alloreactive and autoreactive CLP after alloantigenic stimulation were in the range of 1/100 to 1/4800 and 1/450 to 1/5000, respectively. After coculture with autologous stimulator cells, frequencies of autoreactive and alloreactive DN CLP were 1/700 to 1/2700 and 1/1360 to 1/4500, respectively. Split culture analysis revealed that most proliferating DN colonies selected for high probability of clonality simultaneously killed both autologous and HLA-mismatched allogeneic targets. The majority of the DN cells expressed the $CD3^+/TCR\gamma\delta^+$ phenotype after culture, and thus were not $CD2^+CD3^-$ NK cells. Taken together, our results show that 1) freshly isolated peripheral blood $\gamma\delta^+$ T cells lack cytotoxic activity, and 2) most cytotoxic $\gamma\delta^+$ T cells activated by autologous or allogeneic stimulator cells under limiting dilution conditions do not discriminate between autologous and allogeneic targets.

Most T cells recognize Ag including allogeneic class I or class II MHC molecules via a clonally distributed $\alpha\beta$-TCR heterodimer. In addition, a minor subset of T cells with an alternative TCR composed of γ- and δ-chains has been identified (1). The biologic significance of $\gamma\delta^+$ T cells is still unclear at present. Due to the small number of available V and J gene segments, there is perhaps only limited variability of $\gamma\delta$ as compared to $\alpha\beta$ TCR. However, extensive N region diversity contributing to the $\gamma\delta$-TCR repertoire occurs during the rearrangement of the γ- and δ-chain genes (1–3). Until recently, it has been difficult to demonstrate Ag specificity of $\gamma\delta^+$ T cells. Several groups have reported that certain $\gamma\delta^+$ T cells specifically recognize mycobacterial Ag including in some cases mycobacterial heat shock proteins (4–10). Furthermore, MHC class II-restricted tetanus toxoid-specific $\gamma\delta^+$ clones were described by Kozbor et al. (11). Conventional $\alpha\beta^+$ T cells contain alloreactive precursor cells that respond in high frequency to allelic variants of MHC class I or class II molecules (12, 13), and allo-Ag-specific $\alpha\beta^+$ clones can be easily established (14). In contrast, only few examples of allospecific $\gamma\delta^+$ T cells have been reported to date. Although the allospecificity of a $\gamma\delta^+$ clone could be precisely defined in one case (15), the specificity pattern was less clear in other cases (16–18). It appears that MHC class I-related Ag such as TL in mice and CD1 in man are target Ag for certain $\gamma\delta^+$ T cells (19–21).

In the present study we have performed LD[3] analyses to determine the frequency of alloreactive (and autoreactive) CLP within $\gamma\delta^+$ T cells isolated from human peripheral blood. We show here that LD culture conditions that induce efficient clonal activation of allospecific CLP within E-rosette-separated T cells (22) also stimulate a large fraction of isolated $\gamma\delta^+$ T cells. In contrast to $\alpha\beta^+$ T cells, however, the majority of alloantigen-activated clonally developing $\gamma\delta^+$ CTL is not specific for the stimulating allogeneic HLA Ag.

MATERIALS AND METHODS

Isolation of $\gamma\delta^+$ T cells. PBMC were isolated from buffy coats obtained from healthy HLA-typed blood donors by centrifugation on Ficoll-Hypaque gradients. Plastic-adherent cells were removed by incubating PBMC (2×10^6/ml) for 90 min at 37°C in culture flasks in medium RPMI 1640 supplemented with 10% FCS (Biochrom KG, Berlin, FRG). T cells were purified from plastic-nonadherent PBMC by two cycles of rosette formation with neuraminidase-treated sheep E as described (22). Sheep E were lysed with NH_4Cl solution or, when cells were tested for NK activity, with destilled water. $\gamma\delta^+$ T cells were purified from double E-rosetted T cells as recently described (23). Briefly, 50×10^6 T cells per tube were incubated for 20 min on ice with 1 ml each of OKT4 (anti-CD4) and OKT8 (anti-CD8) hybridoma supernatant. After being washed, the cells were incubated for

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[3] Abbreviations used in this paper: LD, limiting dilution; CLP, cytotoxic lymphocyte precursor; DN, double-negative ($CD4^-CD8^-$) T cell; LCL, lymphoblastoid cell lines.

60 min at 37°C in nontoxic rabbit complement (Cedarlane, Hornby, Canada). Viable cells were recovered by centrifugation on Ficoll-Hypaque. This procedure was repeated once, this time including anti-NK cell mAb Leu 11a (anti-CD16; Becton Dickinson, Mountain View, CA), Leu 19 (anti-CD56), Leu 7 (anti-CD57), and anti-TCR-$\alpha\beta$ mAb BMA 031 (24). Finally, the cells were labeled again with mAb OKT4, OKT8, Leu 11a, Leu 19, and BMA 031, and residual positive cells were depleted by panning on petri dishes coated with goat anti-mouse IgG (Tago, Burlingame, CA) as described by Wysocki and Sato (25). The resulting population of $CD4^-CD8^-$ DN T cells consisted of 82.3 ± 8.2% $\gamma\delta^+$ T cells (10 experiments) as shown by staining with mAb TCR-δ-1 that recognizes a constant epitope on the TCR δ-chain (26). Contamination with $\alpha\beta^+$ T cells was less than 1%.

LD cultures. LD cultures with unseparated T (E^+) and isolated DN responder cells were performed as described (22). Briefly, titrated numbers of E^+ or DN cells were cocultured in 24 replicates with 10^5 irradiated (4000 rad from a cesium source) allogeneic or autologous PBMC stimulator cells in wells of 96-well culture plates (Nunc, Roskilde, Denmark). The culture medium was RPMI 1640 (Biochrom KG) supplemented with 2 mM L-glutamine, 10% heat-inactivated male serum, 10 mM HEPES, antibiotics, and 1 ng/ml human rIl-2 (EuroCetus, Amsterdam, The Netherlands). In some experiments, cells from LD cultures that had a high probability of clonality (27) were further propagated and expanded for 2 to 4 wk in Il-2-supplemented medium. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Establishment of $\alpha\beta^+$ and $\gamma\delta^+$ T cell clones. Il-2-dependent T cell clones were established as we recently reported (28). Briefly, E rosette separated T cells and purified DN cells were cultured at 0.3 cells/well in 96-well round bottom microculture plates in the presence of irradiated PBMC and LCL feeder cells, PHA-P (0.5 μg/ml) and rIl-2 (3 ng/ml). Proliferating clones were expanded in 24-well plates under occasional restimulation with feeder cells and PHA-P.

Cell proliferation and cytotoxicity assay. After 12 to 14 days, LD microcultures were split into three aliquots. To one aliquot, 1 μCi ^{3}H-TdR was added, and incubation at 37°C was continued for 6 h, after which the cultures were harvested and processed for counting of β emission in a Packard scintillation counter (Packard Instruments Inc., Donners Grove, IL). Alternatively, the presence of proliferating cells in individual microcultures was monitored by microscopic inspection. Individual wells of the two remaining aliquots were tested for cytotoxic effector activity against ^{51}Cr-labeled autologous and allogeneic (i.e., stimulator-derived) Con A blast target cells as described (22). Briefly, 3-day Con A blasts (2×10^6) were labeled for 90 min with 350 μCi sodium-^{51}chromate. After being washed, the labeled target cells were added at 1 to 2000 cells per well to LD cultures, and incubation was continued at 37°C. After 4 h, 100 μl of supernatant were removed from each well for counting of γ-emission in a Packard gamma-counter. Spontaneous lysis was determined from wells where no responder cells had been added, and maximal lysis was measured in wells that had been vigorously resuspended. Percentage specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}}}{\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}} \times 100$$

In some experiments, the following established cell lines were used as target cells: NK-susceptible K562, OKT3 hybridoma (American Type Culture Collection, Bethesda, MD), 7A5 hybridoma producing mAb against Vγ9 of the human $\gamma\delta$ TCR (O. Janssen et al., unpublished observations), and EBV-transformed LCL.

Statistical analysis. For determination of frequencies of proliferating lymphocyte precursors and CLP, all wells where the ^{3}H-TdR incorporation or ^{51}Cr-release exceeded the mean value of 24 control wells by at least 3 SD were considered positive. Frequencies of proliferating lymphocyte precursors and CLP were calculated based on the minimum χ^2 method (29) by using a computer program kindly provided by Dr. Heeg (Ulm University, Ulm, FRG).

Immunofluorescence analysis. E^+ and DN responder cells were analyzed before and after culture by two-color immunofluorescence. The following mAb were used as FITC or PE conjugates: Leu 4 (anti-CD3, Becton Dickinson); OKT11 (anti-CD2, Ortho Pharmaceuticals, Raritan, NJ); BMA 031 (anti-TCR-$\alpha\beta$) (24); TiγA (anti-TCR Vγ9 JPCγ1) (30); TCRδ-1 (anti-TCR $\gamma\delta$, T Cell Sciences, Boston, MA), and 7A5 (anti-TCR-$\gamma\delta$ made in our laboratory; staining pattern identical to TiγA) (O. Janssen et al., unpublished observations). Cells (0.5 to 1×10^5) were incubated for 20 min on ice with the appropriate mAb. After being washed twice in PBS/1% BSA/0.1% sodium azide, the samples were resuspended in 1% paraformaldehyde and analyzed in a FACScan cytofluometer (Becton Dickinson).

RESULTS

Freshly isolated $\gamma\delta^+$ T cells lack cytotoxic activity. Established $\gamma\delta^+$ clones frequently exert cytotoxic activity against NK-susceptible target cells (18, 31). In contrast, resting $\gamma\delta^+$ T cells isolated by negative selection from peripheral blood did not kill NK-susceptible K562 cells (Table I). In further contrast to activated $\gamma\delta^+$ T cells, cytotoxic effector function could not be triggered in resting $\gamma\delta^+$ T cells by signalling via the CD3/TCR complex. Hybridomas producing mAb against CD3 or TCR are sensitive targets for CTL (32). As reported in Table I, anti-Vγ9 mAb producing 7A5 hybridoma cells were efficiently killed by Il-2-dependent $\gamma\delta^+$ clones. 7A5 hybridoma cells were resistant, however, to lysis by freshly isolated DN cells consisting of 86% $7A5^+$ cells. 7A5 target cells were killed by TCR-$\gamma\delta^+$ clones but not by either $CD4^+$ or $CD8^+$ TCR-$\alpha\beta^+$ clones (Table II). As expected, all three types of cytotoxic T cell clones killed anti-CD3 mAb producing OKT3 hybridoma cells (see Table II).

Frequency analysis of alloreactive and autoreactive $\gamma\delta^+$ CLP. Previously established LD culture conditions (22) were used to determine the frequency and specificity of $\gamma\delta^+$ CLP proliferating in response to allogeneic or autologous PBMC stimulator cells. To avoid preactivation of $\gamma\delta^+$ T cells as occurring during positive selection using anti-TCR-$\gamma\delta$ mAb and cell sorting, we developed a negative isolation procedure that is based on the depletion of $CD4^+$, $CD8^+$, TCR-$\alpha\beta^+$, $CD16^+$, $CD56^+$, and $CD57^+$ NK cells from E rosette-purified T cells (23). In 10 consecutive experiments, the resulting population of DN T cells consisted of 82.3 ± 8.2% TCRδ-1^+ $\gamma\delta^+$ T cells and <0.5% BMA031$^+$ $\alpha\beta^+$ T cells. This population is referred to as DN or $\gamma\delta^+$ T cells. When the total population of E rosette separated T cells was used as responder population, the expected frequencies of alloreactive or autoreactive CLP were measured. Results of a representative experiment are shown in Figure 1. As seen in Figure 1 (*upper left*), 1 of 184 E^+ cells proliferated in response to allogeneic stimulator cells. Frequencies of CLP as tested against the specific (stimulator-derived) or autologous (responder-derived) Con A blast target were 1/2,364 and <1/20,000, respectively. When E^+ T cells were cocultured with autologous PBMC feeder cells, 1 of 196 cells proliferated, but frequencies of CLP as tested against autologous or allogeneic targets were undetectable ($f < 1/20{,}000$, see Fig. 1, *upper right*). Quite different results were obtained with DN responder cells. As seen in Figure 1 (*lower part*), 1 of 662 DN cells proliferated in response to allogeneic stimulator cells. In this case, the frequency of alloreactive DN CLP was 1/2,143, but a significant fraction of allostimulated DN responder cells also killed autologous Con A blast target cells ($f = 1/5{,}414$). When purified DN cells were cocultured with autologous PBMC stimulator cells, 1 of 116 responder cells proliferated, of which a signifi-

TABLE I
Freshly isolated $\gamma\delta^+$ T cells lack cytotoxic activity

Effector	E/T Ratio	Target (Percent Specific Lysis)	
		K562	7A5
Plastic-nonadherent PBMC	50:1	35.4	0.0
Unseparated T (E^+)	50:1	5.7	0.0
Double-negative T (86% $7A5^+$)	50:1	2.7	0.0
$7A5^+$ clone D730	2:1	ND	56.0

TABLE II

$\gamma\delta^+$ but not $\alpha\beta^+$ CTL clones kill anti-TCR-$\gamma\delta$ mAb producing 7A5 hybridoma target cells[a]

Clone	Phenotype	Percent Specific Lysis			
		OKT3 (E/T)		7A5 (E/T)	
		4:1	2:1	4:1	2:1
A37E+2	$CD4^+CD8^-TCR\text{-}\alpha\beta^+$	19.2	13.1	0.0	0.0
A37E+20	$CD4^-CD8^+TCR\text{-}\alpha\beta^+$	14.9	12.8	0.0	0.0
A37DN8	$CD4^-CD8^-TCR\text{-}\gamma\delta^+$	41.4	32.1	66.1	59.1
A37DN14	$CD4^-CD8^-TCR\text{-}\gamma\delta^+$	22.8	15.1	44.8	33.7
A37DN24	$CD4^-CD8^-TCR\text{-}\gamma\delta^+$	65.9	47.8	67.4	52.2

[a] Results are given as percentage specific lysis of OKT3 and 7A5 hybridoma cells at the indicated E:T ratios.

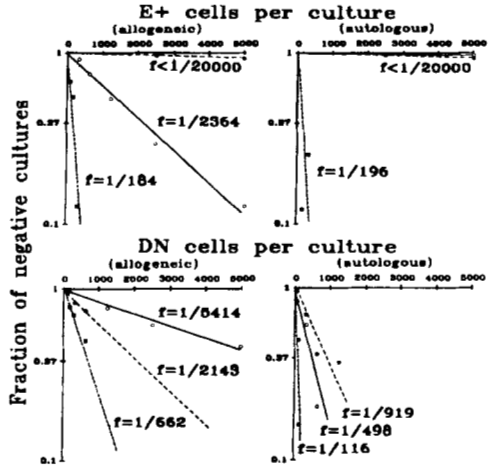

Figure 1. Limiting dilution analysis of allostimulated and autostimulated E^+ and DN responder cells. E^+ (88% $\alpha\beta^+$, 6% $\gamma\delta^+$; *upper part*) and DN (0.5% $\alpha\beta^+$, 74% $\gamma\delta^+$; *lower part*) were cocultured under LD conditions with HLA-mismatched allogeneic (*left*) or autologous (*right*) PBMC stimulator cells. After 13 days, all microcultures were split into three aliquots and tested for ^{3}H-TdR incorporation and cytotoxic activity against specific (allogeneic) or autologous Con A blast targets. The fraction of negative cultures is plotted on the *y axis* vs the number of responder cells seeded per well on the *x axis*. The frequencies of proliferating lymphocytes (■) and of alloreactive (○) and autoreactive (●) CLP are shown. *p* values indicating single hit kinetics (30) were >0.6 in all cases. HLA phenotypes of responder and stimulator were as follows. Responder: A 24,25; B 18,62; C 3,–; DR 2,–. Stimulator: A 2,31; B 7,65; C 7,8; DR 1,11.

cant fraction killed allogeneic (f = 1/498) or autologous (f = 1/919) target cells. HLA phenotypes of the respective responder and stimulator cells are listed in Table III. Results of several additional LD experiments are presented in Tables IV and V. The results can be summarized as follows. When unseparated T cells (E^+) were stimulated with allogeneic PBMC, 1 of 420 to 2300 cells developed into a CTL that specifically lysed stimulator but not autologous Con A blasts (f against autologous targets <1/20,000; Table IV). A significant fraction of E^+ cells proliferated in response to autologous PBMC, but frequencies of autoreactive or alloreactive CLP as tested against both targets were <1/20,000 (Table V). In striking contrast, significant numbers of purified DN cells clonally developed into alloreactive (f = 1/100 – 1/4,800) or autoreactive (f = 1/450 – 1/5,000) CTL when stimulated by allogeneic PBMC under LD culture conditions (Table IV). Importantly, roughly similar CLP frequencies were measured when isolated DN responder cells were cocultured with autologous PBMC stimulator cells. One of 720 to 2700 seeded DN cells killed autologous Con A blasts, and 1 of 1360 to 4500 cells killed HLA class I and class II mismatched allogeneic Con A blasts (Table V).

Specificity of $\gamma\delta^+$ CLP. The above data showed that DN cells clonally proliferate in high frequency in response to autologous or allogeneic PBMC feeder cells. The frequency analyses suggested that the majority of growing $\gamma\delta^+$ CTL colonies did not discriminate between autologous and allogeneic target cells. To investigate the specificity pattern of allo/autostimulated $\gamma\delta^+$ CLP in more detail, individual LD microcultures with a >70% probability of clonality (27) were split into two aliquots and tested against allogeneic (stimulator-derived) and autologous (responder derived) Con A blast targets. As can be seen in Figure 2*A* the majority of colonies developing from E^+ responder cells after alloantigenic stimulation specifically lysed allogeneic (stimulator derived) targets, whereas only a few CTL colonies also lysed autologous (responder) targets. In contrast, the majority of colonies developing from DN responder cells after alloantigenic stimulation lysed both allogeneic and autologous target cells. Only a minor fraction of proliferating DN colonies exerted specific lysis of stimulator targets (Fig. 2*B*). Similarly, DN responder cells cocultured under LD conditions with autologous stimulator cells exerted nonspecific lysis of both autologous and allogeneic target cells (Fig. 2*C*).

The negative selection protocol used to isolate $\gamma\delta^+$ T cells did not yield pure $\gamma\delta^+$ T cells; the resulting DN population was consistently contaminated with 5 to 10% $CD2^+CD3^-$ cells known to include NK cells (33). Therefore, we considered it important to prove that DN cells proliferating in LD cultures were indeed $\gamma\delta^+$ T cells. To this end, individual LD microcultures were split into four

TABLE III

HLA phenotypes of responder and allogeneic stimulator/target cells used in the experiments listed in Tables IV and V

Expt.	HLA Responder				HLA Allogeneic Stimulator/Target			
	A	B	C	DR	A	B	C	DR
A57	3, 31	7, 62	3, 7	1, 2	1, 2	55, 60	1, 3	13, –
A61	1, 24	8, 51	7, –	2, 13	2, 31	7, 65	7, 8	1, 11
A63	1, 24	7, 8	7, –	2, –	2, –	60, 62	3, –	13, –
D831	2, 31	7, 51	2, 7	4, –	1, 11	8, 35	4, 7	3, 13
D840	1, 11	8, 35	4, 7	3, 13	3, –	7, –	7, –	1, –
D848	28, 29	35, 44	4, –	13, 15	3, 24	18, 27	2, –	2, 4
D898	1, 26	39, –	9, –	1, 14	2, 29	7, –	7, –	2, 7

TABLE IV

CLP frequency analysis of allostimulated E^+ and DN responder cells[a]

Expt.	E^+ Responder Cells			DN Responder Cells		
	Proliferation	Allogeneic	Autologous	Proliferation	Allogeneic	Autologous
A57	1/80	1/1,630	<1/20,000	1/190	1/4,800	1/5,000
A61	1/220	1/1,170	<1/20,000	1/760	1/2,470	1/5,000
D840		1/1,700	<1/20,000		1/2,630	1/2,980
D848		1/420	1/12,000		1/880	1/450
D898		1/2,120	<1/20,000		1/105	1/470

[a] E^+ or DN responder cells were cocultured under LD conditions with allogeneic (HLA class I and class II mismatched) stimulator cells. After 12 to 14 days, each well was split into three aliquots. One aliquot was pulsed for 6 h with ^{3}H-TdR for the determination of proliferating lymphocytes. The remaining two aliquots were tested for cytotoxic effector activity against allogeneic (stimulator derived) or autologous Con A blast targets. Frequencies of proliferating and CLP were determined as described in *Material and Methods*. *p* values for single hit kinetics were >0.7 in all experiments.

TABLE V

CLP frequency analysis of autostimulated E^+ and DN responder cells[a]

Expt.	E^+ Responder Cells			DN Responder Cells		
	Proliferation	Allogeneic	Autologous	Proliferation	Allogeneic	Autologous
A57	1/530	<1/20,000	<1/20,000	1/230	1/1,480	1/970
A61	1/2,410	<1/20,000	<1/20,000	1/860	1/3,630	1/2,500
A63	1/640	<1/20,000	<1/20,000	1/60	1/2,600	1/2,700
D831				1/350	1/4,500	1/1,720
D848				1/210	1/1,360	1/720

[a] Legend as for TABLE IV. E^+ and DN responder cells were cocultured with autologous PBMC stimulator cells. *p* values for single hit kinetics were >0.6 in all experiments.

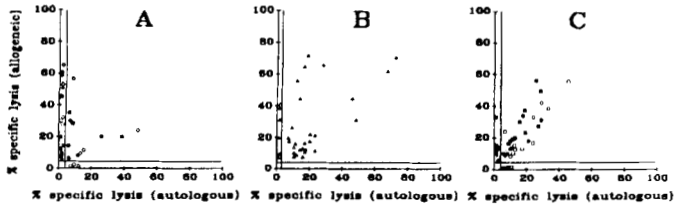

Figure 2. Specificity of clonally proliferating CLP. E^+ (*A*) and DN (*B* and *C*) responder cells were cocultured under LD conditions with allogeneic (*A* and *B*) or autologous (*C*) stimulator cells. After 13 days, individual microcultures were split into two aliquots and tested for specific cytotoxic activity against autologous (*x axis*) and allogeneic (*y axis*) Con A blast targets. At the initiation of culture, microwells were seeded with the following number of responder cells: ■ 625, ● 312, □ 156, ○ 78, △ 39, ▲ 20.

aliquots. Two aliquots were used to measure the cytotoxic effector activity against allogeneic and autologous Con A blast targets, and the remaining two aliquots were used for cell surface marker analysis of proliferating cells. Representative results of an LD experiment where colonies were chosen for high (>70%) probability of clonality are depicted in Figure 3. After alloantigenic stimulation of DN responder cells, 1 of 15 analyzed colonies with a $CD2^+CD3^+TCR\text{-}\gamma\delta(Ti\gamma A)^+TCR\text{-}\alpha\beta(BMA031)^-$ phenotype displayed allospecific cytotoxicity (Fig. 3*A*). The majority (10 of 15) of allostimulated clonally proliferating DN colonies were $CD2^+CD3^+\gamma\delta^+$ $\alpha\beta^-$ and lysed both allogeneic and autologous target cells (Fig. 3*B*). In addition, 3 of 15 proliferating DN colonies with a $CD2^+CD3^+\gamma\delta^+\alpha\beta^-$ phenotype did not kill either allogeneic or autologous Con A blast targets (Fig. 3*C*). In contrast, five of five allostimulated proliferating E^+ colonies displayed allospecific cytotoxicity and an $CD2^+CD3^+\gamma\delta^-\alpha\beta^+$ phenotype as exemplified in Figure 3*D*. Only in rare instances did we detect colonies (1 of 15) with a $CD2^+CD3^-$ phenotype and nonspecific cytolytic activity (Fig. 3*E*). Quantitative similar results were obtained in a second independent experiment. This type of analysis thus ensured that the majority of proliferating DN cells cultured under our LD conditions were indeed $\gamma\delta^+$ T cells.

Finally, we investigated whether the apparent lack of (allo) specificity of alloactivated $\gamma\delta^+$ T cells was restricted to the usage of Con A blast targets or could be also seen with other types of target cells. To this end, total T cells (E^+) and separated DN cells were cocultured under LD conditions with allogeneic and autologous feeder cells. Proliferating cells from wells seeded with low responder cell numbers (<100/well) were expanded for 4 wk in Il-2-supplemented medium. Colonies were then tested for cytotoxic activity at titrated E:T ratios against allogeneic (specific) and autologous EBV-transformed LCL target cells. As shown in Table VI, $\alpha\beta^+$ E^+ cells displayed a high degree of specificity for the stimulator-derived allogeneic LCL. In contrast, $\gamma\delta^+$ DN effector cells did not discriminate between the two targets and efficiently lysed both allogeneic and autologous LCL.

DISCUSSION

The present studies were undertaken to analyze the frequency of cytotoxic lymphocyte precursors within peripheral blood $\gamma\delta^+$ T cells after in vitro activation with allogeneic or autologous PBMC stimulator cells. The LD system used in this study has been previously found to allow reproducible frequency estimations of allospecific CLP within E rosette-separated T cells (22). As shown, identical LD conditions also induced the clonal activation of $\gamma\delta^+$ CLP isolated from peripheral blood. In striking contrast to unseparated (E^+) T cells, however, the majority of proliferating $\gamma\delta^+$ cytotoxic effector cells did not specifically recognize polymorphic MHC Ag.

It has been difficult to assign MHC-restricted Ag specificity to $\gamma\delta^+$ T cells. Several groups including our own have recently reported that $\gamma\delta^+$ T cells frequently respond to mycobacterial Ag (4–10). In addition, HLA-DR-restricted $\gamma\delta^+$ clones were established from a tetanus toxoid-hyperimmune individual by Kozbor et al. (11). To date, only a few reports have addressed the MHC Ag reactivity of $\gamma\delta^+$ T cells. Among these, several studies suggested that $\gamma\delta^+$ T cells might recognize MHC class I-like Ag such as TL in mice (19, 20) and CD1 in man (21). Although Ciccone et al. successfully induced allospecific $\gamma\delta^+$ CTL in MLR (16), alloantigen-specific $\gamma\delta^+$ T cell clones have been established in only a few instances (17, 18).

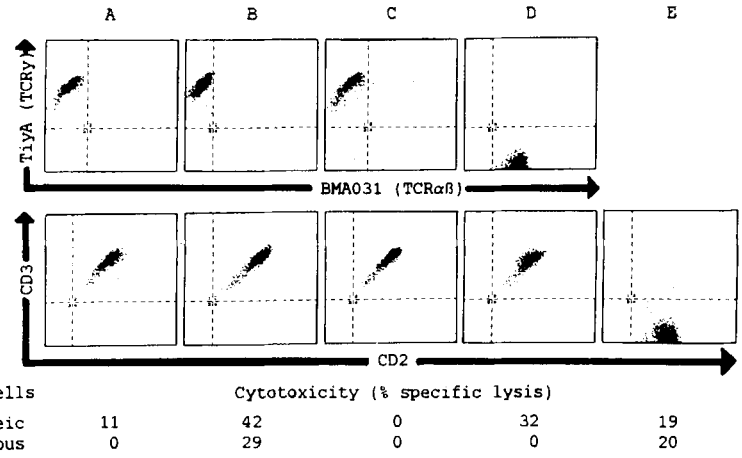

Figure 3. Correlation of phenotype and cytotoxic specificity of clonally proliferating DN CLP. LD cultures were set up with DN (*A, B, C,* and *E*) or E^+ (*D*) responder cells and HLA-mismatched PBMC stimulator cells. After 15 days, individual microcultures selected for a high probability of clonality (i.e., seeded with <70 responder per well) were split into four aliquots. Two aliquots were used for two-color cytofluorographic cell surface analysis (TCR $\gamma\delta$ (*TiγA*) vs TCR $\alpha\beta$ (*BMA 031*), *upper row*; CD3 (Leu 4a) vs CD2 (OKT11), *lower row*). The remaining two aliquots were tested for cytotoxic activity against allogeneic (specific) and autologous Con A blast targets.

TABLE VI

Cytotoxic activity of allo/autostimulated E^+ and DN responder cells against LCL targets[a]

Responder	Stimulator MNC	TCR phenotype	Target					
			Allogeneic LCL			Autologous LCL		
			25:1[b]	12:1	6:1	25:1	12:1	6:1
E^+	Allogeneic	$\alpha\beta^+$	61.7[c]	37.4	22.8	3.3	3.1	1.1
E^+	Allogeneic	$\alpha\beta^+$	47.3	37.5	24.3	0.0	2.4	0.0
E^+	Allogeneic	$\alpha\beta^+$	29.0	19.8	13.1	6.3	0.4	0.9
DN	Allogeneic	$\gamma\delta^+$	58.8	45.1	32.3	29.6	26.9	19.4
DN	Allogeneic	$\gamma\delta^+$	26.1	15.1	11.2	27.4	26.1	19.5
DN	Autologous	$\gamma\delta^+$	68.2	65.6	53.2	46.6	42.6	25.4
DN	Autologous	$\gamma\delta^+$	53.3	50.7	46.4	37.1	30.4	20.4

[a] Microcultures of allo- or autostimulated E^+ and DN responder cells with a >70% probability of clonality were expanded for 4 wk in IL-2-supplemented medium. The cytotoxic activity was then tested against allogeneic (stimulator-derived) or autologous (responder-derived) LCL target cells at the indicated E/T ratios. The TCR phenotype of effector cells was determined using BMA031 ($\alpha\beta$) and TCR-δ1 ($\gamma\delta$) mAB. The HLA-phenotype of responder and stimulator was as follows. Responder: A1, 3; B18, 62; C3/–; DR3/4. Stimulator: A2/–; B39, 51; C–/–; DR2, 5.

[b] E/T ratio.

[c] Percent specific lysis.

The success of Ciccone et al. (16) to generate allospecific $\gamma\delta^+$ CTL in conventional MLR is not necessarily in contrast to our results. In their experiments, Ciccone et al. (16) used bulk culture conditions with 50,000 responder cells per microculture in the absence of exogenous Il-2, whereas LD culture conditions in the presence of added Il-2 were used in our studies. It is thus possible that Ciccone et al. (16) detected the progeny of only one or a few allospecific $\gamma\delta^+$ CLP per microculture well, thus resulting in an estimated frequency of allospecific $\gamma\delta^+$ CLP that might be well in line with our data. More recently, Ciccone et al. (15) identified HLA-A24 as a target Ag for a human $\gamma\delta^+$ clone. Our present results would suggest that only a minor fraction of peripheral blood $\gamma\delta^+$ T cells can give rise to alloantigen-specific CTL. The majority of $\gamma\delta^+$ CLP activated under LD conditions by HLA-mismatched stimulator cells killed both specific stimulator and responder (i.e., autologous) targets (Fig. 2*B*). Therefore, we would speculate that alloantigen-specific $\gamma\delta^+$ lines and clones such as the ones established by Ciccone et al. (15, 16) must originate from precursors that represent only a minority of alloantigen-stimulated $\gamma\delta^+$ CLP (compare Fig. 2*B*).

To avoid a possible preactivation of $\gamma\delta^+$ T cells as occurring during positive selection with anti-TCR mAb, we isolated $\gamma\delta^+$ T cells by depleting E rosette separated T cells of $CD4^+$, $CD8^+$, $CD16^+$, $CD56^+$, $CD57^+$, and TCR-$\alpha\beta^+$ cells by C′-mediated lysis and panning. The resulting population of DN T cells lacked NK activity as tested against K562 target cells and did not lyse anti-TCR-$\gamma\delta$ mAb-producing hybridoma cells. It is thus clear that cytolytic activity is a feature of activated but not resting $\gamma\delta^+$ T cells (34). Despite the efficient negative enrichment of $\gamma\delta^+$ T cells (82.3 ± 8.2%), the resulting cell population was contaminated with 5 to 10% $CD2^+CD3^-$ cells. Therefore, we were concerned that the seemingly nonspecific cytolytic activity of DN cells in our LD cultures might be mediated by $CD2^+CD3^-$ NK cells rather than $CD3^+$ $\gamma\delta^+$ T cells. To address this possibility, two-color immunofluorescence analyses were performed on alloantigen-stimulated proliferating DN cells after culture. These analyses revealed that the majority of clonally proliferating DN cells was indeed TCR $\gamma\delta^+$ (Fig. 3). We conclude that the "nonspecific" promiscuous cytotoxicity after coculture with allogeneic or autologous PBMC stimulator cells was exerted by $\gamma\delta^+$ CTL.

LD culture systems are critically dependent on the exogenous supply of required growth factors. To ensure an efficient clonal proliferation of Ag-triggered responder cells, LD cultures were supplemented with 1 ng/ml rIl-2. These conditions are suitable for the induction of allospecific CLP from E rosette separated T cells (22). Very high concentrations of Il-2 are known to induce promiscuous nonspecific cytotoxicity in Ag-specific $\alpha\beta^+$ T cells (35). It is possible that the specificity pattern of $\gamma\delta^+$ CTL is more susceptible to modulation by cytokines as compared to $\alpha\beta^+$ T cells and, in consequence, that the addition of exogenous Il-2 might contribute to the apparent lack of alloantigen specificity of $\gamma\delta^+$ CLP in our LD cultures. In this context it is of interest that Il-4 was found to

counteract the Il-2-induced non-MHC-restricted cytotoxicity against K562 of a human $\gamma\delta^+$ T cell clone (31). A detailed analysis of the impact of various cytokines (Il-2, Il-4, Il-7, IFN-γ, TNF-α) on the specificity pattern of established $\gamma\delta^+$ T cell clones is currently being performed in our laboratory.

In conclusion, we present the first frequency analysis of peripheral blood TCR $\gamma\delta^+$ T cells that respond to HLA-mismatched allogeneic or autologous stimulator cells. Our results show that LD culture conditions that are suitable for the clonal activation of allospecific $\alpha\beta^+$ CLP also induce clonal growth of $\gamma\delta^+$ CLP. In contrast to $\alpha\beta^+$ CTL, however, the majority of clonally developing $\gamma\delta^+$ CTL does not discriminate between allogeneic and autologous target cells. Therefore, our results would support the contention that $\gamma\delta^+$ T cells differ from $\alpha\beta^+$ T cells in that the majority of $\gamma\delta^+$ T cells is not destined to recognize classical allelic MHC class I or class II Ag.

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