

A LARGE FRACTION OF HUMAN PERIPHERAL BLOOD
 γ/δ^+ T CELLS IS ACTIVATED BY
MYCOBACTERIUM TUBERCULOSIS BUT NOT BY
ITS 65-kD HEAT SHOCK PROTEIN

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Most T cells recognize foreign antigenic peptides bound to self-MHC class I or class II molecules via a clonally distributed TCR- α/β heterodimer (1). A second type of TCR molecule composed of a γ/δ heterodimer has recently been discovered (2-4). γ/δ^+ T cells appear early during fetal thymic ontogeny (4-6). In the adult mouse, γ/δ^+ T cells are a minor population in the periphery (spleen and blood) but appear to be locally enriched in the epidermis and intestinal epithelium (4, 7, 8). In man, γ/δ^+ T cells account for 1-5% of peripheral blood T cells, and they are evenly distributed throughout the lymphoid organs and skin- and gut-associated lymphoid tissues, with no discernible local preference (4, 9, 10). The physiological significance of γ/δ^+ T cells remains unclear (11, 12). Due to the relatively small number of V_γ , J_γ , V_δ , and J_δ segments present in the human genome, there is perhaps only limited diversity of TCR- γ/δ molecules, in contrast to α/β^+ T cells, where diversity is generated from selection among a large number of available V_α , J_α , V_β , and J_β segments (1, 11). Therefore, it is envisaged that γ/δ^+ T cells can recognize only a limited range of foreign antigens via their TCR molecule. In fact, until recently, it has been notoriously difficult to demonstrate MHC-restricted antigen specificity of γ/δ^+ T cells. Only a few examples of MHC class I-specific or foreign antigen (tetanus toxoid)-specific, MHC class II-restricted γ/δ^+ T cells have been described to date (13-17). The limited TCR diversity together with the local prevalence in certain species led to the hypothesis that γ/δ^+ T cells represent a phylogenetically very old surveillance system to monitor cell integrity and to destroy cells altered by transformation or invading microorganisms (18).

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¹ *Abbreviations used in this paper:* DN, double-negative ($CD4^-CD8^-$) T cells; *f*, frequency; hsp, heat shock protein; LCL, lymphoblastoid cell line; LD, limiting dilution; *M. tb*, *Mycobacterium tuberculosis*; PE, phycoerythrin; PPD, purified protein derivative of *M. tuberculosis*; SEE, staphylococcal enterotoxin E.

Evidence for a role of γ/δ^+ T cells in the immune response to mycobacteria has been reported recently by several groups. γ/δ^+ T cells specific for mycobacterial antigens have been isolated from a patient with rheumatoid arthritis (19), from a normal purified protein derivative (PPD)¹-immune individual (20), and from certain lepromatous granulomas (21). In addition, γ/δ^+ T cells were shown to accumulate in draining lymph nodes of mice immunized with *Mycobacterium tuberculosis* (*M. tb*) (22), and a high percentage of murine γ/δ^+ T cell hybridomas was found to recognize *M. tb* antigens (23). In some but not all studies, a bacterial heat shock protein of 65 kD (hsp 65) was identified as a ligand (19, 20, 23).

In the present study, we have used a limiting dilution (LD) culture system to quantitate the frequencies of γ/δ^+ T cells responsive to killed *M. tb* organisms and to hsp 65 of *M. tb/M. bovis* in normal individuals with negative tuberculin skin test. We show here that 1 of 2-19 freshly isolated γ/δ^+ T cells gives a proliferative response to killed mycobacteria, while only a minor fraction is activated by the recombinant 65-kD hsp. Established clones of mycobacteria-reactive γ/δ^+ T cells specifically recognized killed mycobacteria, but neither PPD nor 65-kD hsp. These data indicate that antigenic components of *M. tb* other than hsp 65 are highly stimulatory for human γ/δ^+ T cells.

Materials and Methods

Isolation of γ/δ^+ T Cells. Heparinized peripheral blood was obtained from healthy individuals after informed consent. Ficoll-Hypaque-separated PBMC were depleted of plastic-adherent cells by incubation for 90 min at 37°C in RPMI 1640/10% FCS. T cells (E^+) were isolated from nonadherent PBMC by two consecutive steps of rosetting with neuraminidase-treated sheep erythrocytes (24). E^+ cells consisted of >98% CD2⁺ and >92% CD3⁺ cells. To enrich γ/δ^+ T cells from E^+ cells, we took advantage of the fact that most γ/δ^+ T cells lack both CD4 and CD8 antigens (double negative [DN]) (25). DN cells highly enriched for γ/δ^+ T cells were purified by sequential steps of complement-mediated cell lysis and "panning," as recently described (Bender, A., and D. Kabelitz, manuscript submitted for publication). Briefly, E^+ cells were incubated for 30 min on ice with saturating amounts of OKT4 (anti-CD4) plus OKT8 (anti-CD8) hybridoma supernatants. After being washed, the cells were incubated for 45 min at 37°C in nontoxic rabbit complement (Cedarlane, Ontario, Canada), and then they were centrifuged over Ficoll-Hypaque gradients. Viable cells recovered from the interphase were exposed to a second cycle of treatment with mAb plus C'; in addition to OKT4 and OKT8, Leu-11b (anti-CD16; Becton Dickinson & Co., Mountain View, CA) and BMA 031 (anti-TCR- α/β ; Behringwerke, Marburg, FRG) were included to remove CD2⁺CD3⁻ NK cells and residual α/β^+ T cells. As a final step, Ficoll-Hypaque-separated viable cells were incubated once more with mAbs OKT4, OKT8, Leu-11b, and BMA 031. After being washed twice in PBS/1% FCS, the cells were centrifuged in 6-well culture plates (Greiner, Nürtingen, FRG) coated with 20 μ g F(ab')₂ goat anti-mouse IgM + IgG (Tago Inc., Burlingame, CA) and incubated for 45 min at 4°C (26). Nonadherent cells were washed and incubated overnight in RPMI 1640/10% AB serum at 37°C/5% CO₂ before cell surface markers were analyzed by staining with fluorochrome-labeled mAbs. The described procedure allowed for efficient "negative" enrichment of γ/δ^+ T cells (see Results) with no risk of preactivation that might take place during positive selection with anti-TCR- γ/δ mAbs and FACS cell sorting.

Cell Cultures. E^+ and DN cells were cultured in U- or V-shaped 96-well culture plates (Nunc, Roskilde, Denmark), together with 0.5-1 $\times 10^5$ irradiated (4,000 rad) autologous PBMC feeder cells and various antigens/mitogens, as indicated in Results. The culture medium was RPMI 1640 (Biochrom KG, Berlin, FRG) supplemented with 10% heat-inactivated male A⁺ or AB⁺ serum, 2 mM L-glutamine, 10 mM Hepes, and antibiotics (100 U penicillin plus 100 μ g streptomycin per ml). Human rIL-2 (kindly donated by EuroCetus, Amsterdam, The

Netherlands) was added at 1–2 ng/ml, where indicated. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 4–9 d, cultures were pulsed with 1 μ Ci [³H]TdR (sp act, 6.7 Ci/mmol) per well and incubated for another 6 h, after which the cultures were harvested onto filter papers and processed for counting of β emission in a liquid scintillation counter (Packard Instrument Co. Inc., Downers Grove, IL). Results are expressed as mean cpm \pm SD of triplicate cultures.

Limiting Dilution Cultures. To determine frequencies of antigen (and PHA)-reactive lymphocyte precursors, E⁺ and DN responder cells were cultured at titrated cell numbers in 16–24 replicates in V-shaped microtiter plates in the presence of 5×10^4 irradiated autologous PBMC feeder cells per well. LD cultures were supplemented with antigens (*M. tb*, PPD, hsp 65; each at 5 μ g/ml) or PHA-P (0.5 μ g/ml; Wellcome Diagnostics, Burgwedel, FRG) and 1 ng/ml rIL-2. After 12–14 d, all cultures were pulsed with 1 μ Ci [³H]TdR and processed as above. Frequencies (f) of proliferating T cells were estimated by plotting the percentage of negative wells against the responder cell number according to Poisson distribution and by the minimum χ^2 method (27). Frequencies, 95% confidence intervals, and *p* values for single-hit kinetics were calculated with a computer program based on statistical methods of Taswell (27) and kindly provided by Dr. Heeg, Ulm University. In each experiment, 16–24 control wells containing feeder cells, antigen/mitogen, rIL-2, but no responder cells, were set up. All cultures where [³H]TdR uptake exceeded the mean cpm of control cultures by 3 SD were considered positive and used for determination of frequency estimates.

Cloning of *M. tb*-reactive γ/δ^+ T Cells. γ/δ^+ T cell clones were established from LD cultures of *M. tb*-stimulated DN responder cells from two normal individuals with negative tuberculin skin test. Cells were collected from wells where DN responder cells (16 or 32/well) had been cultured for 14 d in the presence of *M. tb* and rIL-2; these cells were cloned at 0.3 cells per well in round-bottomed 96-well culture plates in the presence of 10^5 irradiated autologous PBMC feeder cells, 5 μ g/ml killed mycobacteria, and 2 ng/ml rIL-2. Growing clones were expanded in 24-well culture plates in rIL-2-containing medium. Established clones were restimulated every 2–3 wk with PHA (0.5 μ g/ml) and irradiated allogeneic feeder cells (10^6 PBMC/ml plus 10^5 EBV transformed B cell lines/ml) as described (28). Due to shortage of autologous PBMC, this protocol was used to propagate established clones; it did not have any appreciable effect on the specificity pattern of the clones.

Cell Surface Marker Analysis. For simultaneous two-color cytofluometry, the following mAbs were used as FITC or phycoerythrin (PE) conjugates. Leu-4 (anti-CD3) and Leu-2a (anti-CD8) were from Becton Dickinson & Co., and OKT4 (anti-CD4) was from Ortho Pharmaceutical (Raritan, NJ). TCR- δ -1 directed against a common epitope on the TCR δ chain (29) was purchased from T Cell Sciences (Cambridge, MA), T γ A recognizing a V γ 9J μ C γ 1 epitope (30) was kindly provided by Dr. F. Triebel (Institut Gustave Roussy, Villejuif, France). Biotin-conjugated mAb BMA 031 (anti-TCR- α/β) was visualized using PE-labeled streptavidin (Becton Dickinson & Co.) as a second-step reagent. Cells were incubated for 20 min on ice with the appropriate mAbs, washed twice with PBS/1%FCS/0.1% sodium azide, and resuspended in 1% paraformaldehyde. All samples were analyzed on a FACScan cytofluometer (Becton Dickinson & Co.).

Mycobacteria and Antigens. *M. tb* organisms were grown in Dubos Middlebrook medium supplemented with BSA and Tween 80 at 37°C under heavy shaking. After washing, bacteria were killed and sonified three times for 5 min. Alternatively, lyophilized and killed *M. tb* strain H37Ra (Difco Laboratories, Inc., Detroit, MI) was used after sonication. PPD was obtained from Statens Serum Institute, Copenhagen (Denmark). Recombinant 65-kD hsp of *M. bovis* (fully identical with its homologue in *M. tb*) was produced, isolated, and purified from *Escherichia coli* clone M 1103 (31), as described (32). Staphylococcal enterotoxin type E (SEE) was purchased from Serva (Heidelberg, FRG) and was used at 1 ng/ml.

Restimulation of Mycobacteria-reactive γ/δ^+ Clones. Established clones (see above) were rechallenged at least 10 d after addition of feeder cells. To this end, Ficoll-Hypaque-purified clone cells (3×10^4 /well) were cocultured with feeder cells (3×10^4 or 10^5) in the presence or absence of mycobacterial antigens. The culture medium was RPMI 1640/10% AB serum, without addition of exogenous IL-2. After 24 and 48 h, 75 μ l of supernatant was removed from each well for determination of IL-2 content on IL-2-dependent murine CTLL cells as

described (28). The remaining cells were pulsed for 6 h with [³H]TdR. Feeder cells were irradiated (4,000 rad) autologous or HLA-DR matched/mismatched PBMC, irradiated (8,000 rad) autologous EBV-transformed B cells, or irradiated (8,000 rad) leukemic MOLT-4 cells.

Results

Solubilized M. tb Preferentially Activates γ/δ^+ T Cells. Double E rosette-purified T cells were cultured with autologous PMBC feeder cells and PHA or *M. tb*, or with allogeneic stimulator cells. [³H]TdR incorporation and the TCR phenotype of cultured cells were determined after 6–9 d. As reported in Table I, killed mycobacteria induced a strong proliferative response in E⁺ cells, comparable in magnitude with PHA or allogeneic MLR stimulation. In marked contrast to PHA and MLR cultures, however, mycobacteria preferentially activated γ/δ^+ T cells. Freshly isolated E⁺ responder cells consisted of 85–92% α/β^+ and 3–7% γ/δ^+ cells, as revealed by staining with mAbs BMA 031 (anti-TCR- α/β) and TCR- δ -1 (anti-TCR- δ). While this low percentage of γ/δ^+ T cells did not change significantly upon culture in PHA or MLR, a dramatic increase of γ/δ^+ cells was noted in *M. tb*-stimulated cultures. Thus, from 25 to 52% of mycobacteria-activated responder cells expressed the TCR- γ/δ after a culture period of 6–9 d (Table I). The potent stimulatory activity of killed mycobacteria on γ/δ^+ T cells was confirmed when purified DN cells (highly enriched for γ/δ^+ T cells) were used as responders. As shown in Table II, the proliferative response to mycobacteria was several-fold increased in DN cells (77.7% γ/δ^+) as compared with E⁺ cells (6.9% γ/δ^+), provided the cultures were supplemented with exogenous IL-2. Note that in this experiment, only 3,000 responder cells were seeded per microculture well. Under these conditions, exogenous rIL-2 was required to reveal the stimulatory capacity of mycobacteria (see Table II). As compared with killed mycobacteria, both PPD and the recombinant hsp 65 were only weakly stimulatory (both for E⁺ and DN responder cells).

Frequencies of M. tb-reactive T Cells. The above data suggested that killed mycobacteria stimulate γ/δ^+ T cells from normal donors much more efficiently than does either PPD or hsp 65. In fact, the results presented in Tables I and II indicate that mycobacteria and PHA exert comparable stimulatory activity on DN responder cells. To

TABLE I
M. tb Preferentially Activates γ/δ^+ T Cells

Stimulus	Exp. 1 (day 6)			Exp. 2 (day 8)			Exp. 3 (day 9)	
	α/β^+	γ/δ^+	[³ H]TdR *	α/β^+	γ/δ^+	[³ H]TdR	α/β^+	γ/δ^+
	%	%	cpm	%	%	cpm	%	%
–	90.6	4.2	–	92.4	3.8	–	85.3	7.1
PHA	93.1	5.3	54,358	90.8	1.5	18,415	94.7	2.4
MLR	78.5	2.9	48,748	79.8	2.2	30,064	81.7	6.4
<i>M. tb</i>	59.2	34.2	59,138	65.5	25.4	37,633	42.0	52.0

0.5–1 × 10⁵ double E rosette-purified T cells were cultured in quadruplicate with irradiated autologous PBMC feeder cells in the presence of PHA or mycobacteria (*M. tb*), or with irradiated allogeneic PBMC stimulator cells (MLR). After 6–9 d, one aliquot of each culture was stained with BMA 031-PE (TCR- α/β) plus TCR- δ -1-FITC (TCR- δ) and analyzed on a FACScan. The remaining three wells of each culture were pulsed for 6 h with [³H]TdR.

* Mean cpm of triplicate cultures; SD was always <15%.

TABLE II
Proliferative Response of E⁺ and DN T Cells to *M. tb*

Stimulus <i>μg/ml</i>	rIL-2 (3 ng/ml)	[³ H]TdR Uptake	
		E ⁺ cells (6.9% γ/δ^+)	DN cells (77.7% γ/δ^+)
<i>cpm</i> \pm SD			
-	-	189 \pm 32	270 \pm 12
PHA (1)	-	60,041 \pm 929	36,009 \pm 3,712
<i>M. tb</i> (5)	-	5,010 \pm 1,082	1,767 \pm 85
PPD (5)	-	473 \pm 159	203 \pm 25
hsp 65 (5)	-	214 \pm 83	145 \pm 14
SEE (1)	-	46,843 \pm 7,024	9,912 \pm 1,390
-	+	2,424 \pm 662	2,523 \pm 219
PHA	+	72,119 \pm 6,425	45,612 \pm 3,983
<i>M. tb</i>	+	13,619 \pm 600	50,130 \pm 5,806
PPD	+	5,305 \pm 1,478	12,462 \pm 527
hsp 65	+	4,465 \pm 1,074	8,380 \pm 817
SEE	+	47,979 \pm 2,578	15,027 \pm 201

3,000 E⁺ or DN responder cells were cocultured with irradiated autologous PBMC feeder cells in the presence of mitogens or antigens and rIL-2 as indicated. [³H]TdR incorporation was measured after 5 d. Mean cpm \pm SD of triplicate cultures are given.

more precisely determine the responsiveness of γ/δ^+ T cells to mycobacteria, hsp 65, or PHA, respectively, we performed LD analyses to measure frequencies of responsive cells within E⁺ and DN responder cells. Results of a representative experiment are presented in Fig. 1, *a* and *b*. While both E⁺ (Fig. 1 *a*) and DN cells (Fig. 1 *b*) responded well to PHA ($f = 1/1.7$ and $1/2.2$, respectively), only DN cells responded in equally high frequency ($f = 1/2.4$) to *M. tb* (H37). Of interest, the frequency of DN cells responsive to the recombinant 65-kD hsp was much lower ($f = 1/64$).

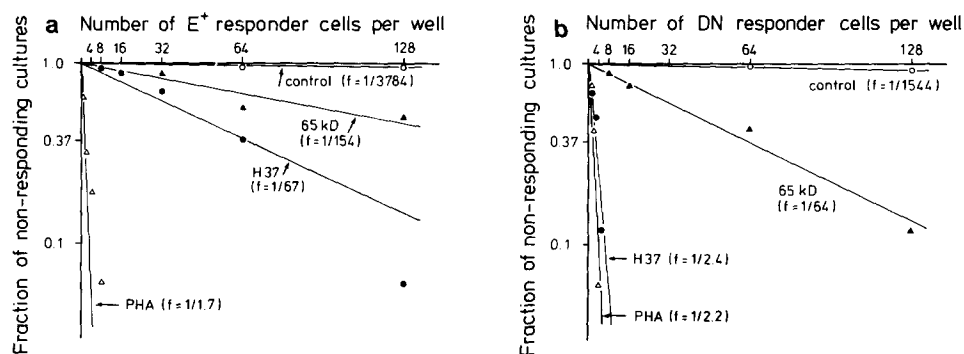


FIGURE 1. Frequency analysis of *M. tb*-reactive E⁺ and DN responder cells. E⁺ (*a*) and DN (83% γ/δ^+ ; *b*) T cells were cultured under LD conditions with autologous PBMC feeder cells in the absence (control) or presence of PHA, *M. tb* organisms (H37), or hsp 65 (65 kD). After 14 d, all microcultures were pulsed with [³H]TdR. The number of responder cells per well is plotted on the x axis vs. the logarithm of the fraction of negative cultures on the y axis. Frequencies (f) of proliferating cells were calculated according to Taswell (27); p values indicative of single-hit kinetic were >0.7 for all frequency estimates.

Note that for both E⁺ and DN responder cells, measured frequencies reflected antigen-triggered T cell activation, since the frequencies of proliferating cells cultured in the absence of antigen/mitogen (i.e., only in the presence of feeder cells and rIL-2) were orders of magnitude lower (Fig. 1). Table III summarizes the results of several additional LD analyses of E⁺ and DN responder cells stimulated with PHA or *M. tb*. It is clear from these data that DN cells are greatly enriched for precursors that can be clonally activated by killed mycobacteria. On average, frequencies of mycobacteria-responsive DN cells were only twofold lower than frequencies of PHA-reactive DN cells, while 15–85-fold differences in frequencies between PHA- and mycobacteria-reactive E⁺ responder cells were noted. Although DN cells were highly enriched for γ/δ^+ cells (65–93% in 10 experiments), we wanted to be sure that DN cells proliferating in response to *M. tb* were indeed γ/δ^+ . Therefore, DN cells cultured with mycobacteria under LD conditions were stained after 12–14 d with BMA 031 and TCR- δ -1. As can be seen in Fig. 2, >98% of mycobacteria-stimulated DN cells were γ/δ^+ . Taken together, these results demonstrate that a major fraction of peripheral blood γ/δ^+ T cells is activated by components of *M. tb*, whereas only a minor population is stimulated by recombinant hsp 65 of mycobacteria.

Characterization of M. tb-reactive γ/δ^+ T Cell Clones. To characterize the specificity pattern of mycobacteria-reactive γ/δ^+ T cells at the clonal level, we established several IL-2-dependent clones. To this end, DN responder cells were cultured under limiting dilution conditions with mycobacteria and autologous PBMC feeder cells. After 14 d, cells from individual microculture wells (seeded with 16 and 32 responder cells) were cloned at 0.3 cells per well in the presence of fresh irradiated PBMC feeder cells, mycobacteria, and rIL-2. Growing clones were expanded in rIL-2-containing medium and were maintained by occasional rechallenge with irradiated feeder cells and PHA (see Materials and Methods). The specificity of established clones was tested by restimulation in the presence of autologous or allogeneic feeder cells and various antigens. Results obtained with two representative clones from one individual are presented in Figs. 3 and 4. Both clones were TCR- δ -1⁺, T γ A⁺ (30) and BMA

TABLE III
*Frequencies of Proliferating E⁺ and DN Responder Cells
Stimulated with PHA or M. tb*

Exp.	E ⁺				DN			
	γ/δ	Frequency			γ/δ	Frequency		
		Control	PHA	<i>M. tb</i>		Control	PHA	<i>M. tb</i>
	%				%			
1	3.3	1/106	1/1.3	1/33	73.4	1/48	1/8.3	1/19
2	2.0	1/562	1/2.4	1/204	63.5	1/49	1/2.3	1/3.2
3	3.5	1/179	1/2.5	1/70	83.8	1/283	1/10	1/19
4	1.0	1/258	1/2.2	1/128	79.4	1/890	ND	1/11
5	14.0	1/45	1/2.3	1/25	70.0	1/54	1/4.4	1/7.6

E⁺ and DN responder cells were cultured under LD conditions with 5×10^4 irradiated autologous PBMC feeder cells in the absence (control) or presence of PHA or mycobacteria (*M. tb*). All cultures were supplemented with 1 ng/ml rIL-2. After 12–14 d, individual microcultures were pulsed with [³H]TdR, and frequencies (*f*) of proliferating cells were calculated as detailed in Materials and Methods. *p* values were >0.6 for all frequency estimates listed in the table.

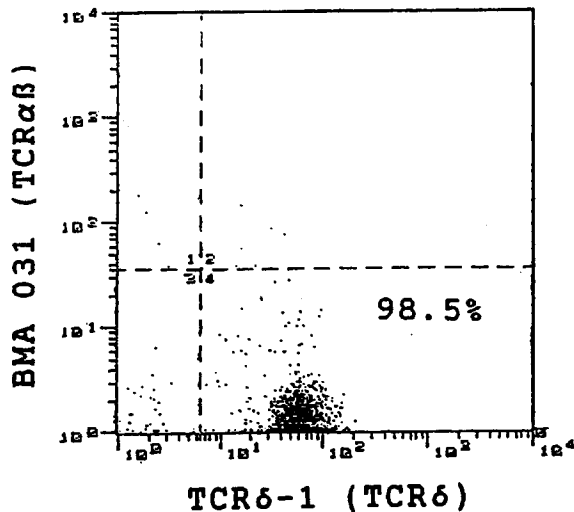


FIGURE 2. TCR phenotype of DN responder cells cultured with *M. tb*. DN responder cells (71% γ/δ^+) were cultured at 32 cells per well with autologous PBMC feeder cells and *M. tb*. After 12 d, the cells were stained with BMA 031-PE plus TCR- δ -1-FITC and analyzed on a FACScan.

031⁻ (not shown). Clone II and clone III proliferated well in response to mycobacteria, but not in response to PPD or hsp 65 (Fig. 3, *a* and *b*). Of interest, both clones required the presence of PBMC feeder cells. No mycobacteria-specific response was seen with autologous EBV-transformed lymphoblastoid cell line (LCL) feeder cells or MOLT-4 leukemic feeder cells. Similar results were obtained with three additional clones (phenotypes: BMA 031⁻, TCR- δ -1⁺, T γ A⁺) from a second individual. Although the clones described here showed a specific response upon rechallenge with mycobacteria in the absence of added IL-2, no endogenously produced IL-2 could be detected in the supernatants (not shown).

To address the question of a possible MHC restriction of *M. tb*-reactive γ/δ^+ clones, restimulation with mycobacteria was performed in the presence of allogeneic PBMC feeder cells that did or did not share HLA-DR antigens with the responder. As shown in Fig. 4, a mycobacteria-specific proliferative response of γ/δ^+ clone III was seen when allogeneic feeder cells shared one DR allele (DR3 or DR7) with the responder, but also when feeder cells lacked DR antigens of the responder (DR3,7). Another mycobacteria-reactive γ/δ^+ clone derived from a DR1,6-positive individual could be restimulated by killed mycobacteria in the presence of DR2,7-positive PBMC feeder cells (not shown). Therefore, we conclude that the established γ/δ^+ T cell clones are mycobacteria specific but not HLA-DR restricted. However, our data do not exclude genetic restriction by other HLA gene products.

Discussion

The present study shows that *M. tb* organisms are potent activators of human peripheral blood γ/δ^+ T cells. Stimulation of unfractionated E⁺ cells (containing 1-7% γ/δ^+ and 80-92% α/β^+ T cells) with mycobacteria led to a selective expansion of γ/δ^+ T cells that comprised 25-52% of all T cells after 6-9 d of culture. In contrast, no preferential activation of γ/δ^+ T cells was seen when E⁺ cells were stimulated with PHA or allogeneic stimulator cells. Mycobacteria-responsive cells were

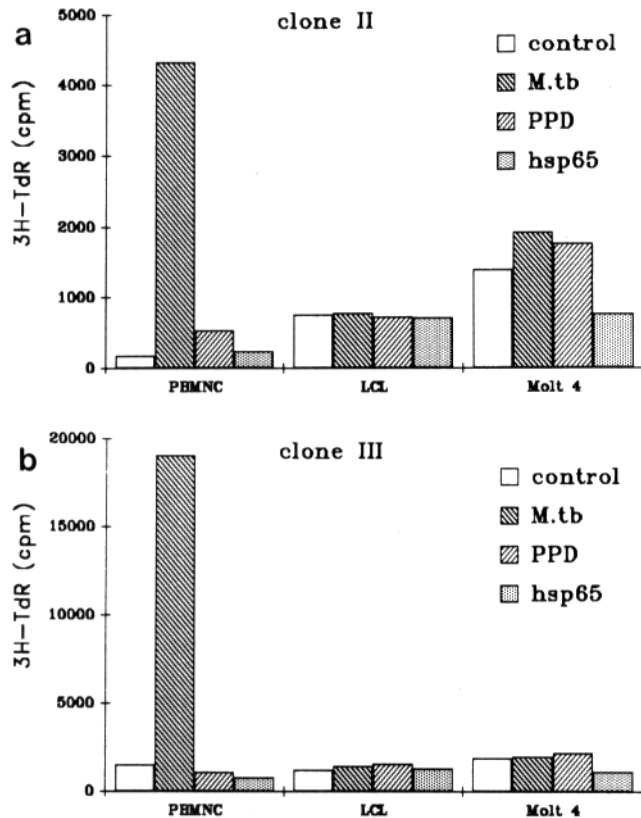


FIGURE 3. Specificity of *M. tb*-reactive γ/δ^+ T cell clones. 3×10^4 γ/δ^+ cells (clone II, a; clone III, b) were cultured in the presence of irradiated autologous PBMC (10^5), autologous EBV-transformed LCL (3×10^4), or MOLT-4 feeder cells (3×10^4) with or without antigens as indicated. [^3H]TdR uptake was determined after 48 h. Mean cpm of triplicate cultures are shown.

enriched within CD4⁻CD8⁻ DN T cells (containing 65–92% γ/δ^+ cells). As judged from [^3H]TdR incorporation, the stimulation of γ/δ^+ T cells by mycobacteria was comparable in magnitude with the mitogenic activation by PHA. To more precisely define the in vitro response to mycobacteria, we applied the LD approach to measure frequencies of mycobacteria-reactive progenitors within E⁺ and DN responder cells. These experiments revealed that 1 of 2–19 DN cells was clonally activated by *M. tb*, while only 1 of 25–200 E⁺ cells proliferated in response to mycobacteria. Within DN responder cells, frequencies of mycobacteria-reactive cells were in the same order (Fig. 1), or only twofold lower (Table III), than frequencies of PHA-reactive cells. In contrast, 15–85-fold differences were noted with E⁺ responder cells.

The T cell response to mycobacterial antigens appears to be complex. With the recent availability of recombinant proteins from *M. tb* (expressed as fusion proteins in *E. coli*; reference 31), it has become possible to study the T cell response to defined epitopes. Among the various proteins identified so far, the 65-kD hsp is of major interest because of the large degree of homology to members of the hsp 60 family found in other prokaryotic and eukaryotic species (33). Hsp 65, as well as other recombinant *M. tb* proteins (12, 19, and 71 kD) elicit an in vitro proliferative T cell response in healthy individuals (34). CD4⁺ T cell clones with specificity for the 65-kD protein of *M. tb* or *M. bovis* have been derived from patients with tuberculosis,

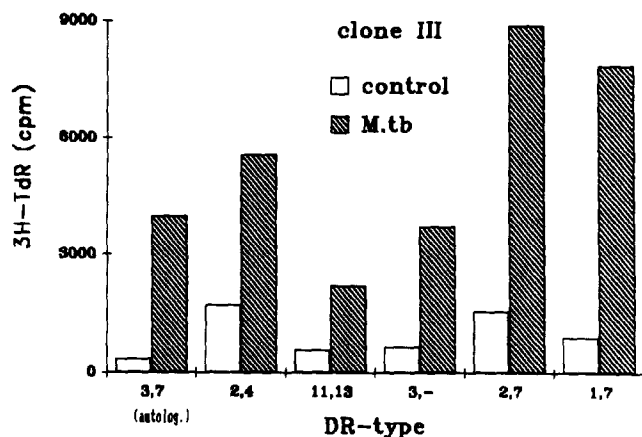


FIGURE 4. *M. tb*-reactive γ/δ^+ T cell clone III is not HLA-DR-restricted. Cloned T cells (3×10^4) were cultured in the presence or absence of *M. tb* with 10^5 irradiated PBMC feeder cells obtained from HLA-typed donors. [^3H]TdR uptake was determined after 48 h. The HLA-DR phenotype of the responder was DR3,7.

tuberculoid leprosy, and healthy PPD-immune donors (35–37). Although no information was provided in these studies, it can be assumed that most of these clones were TCR- α/β^+ , because γ/δ^+ T cells generally lack CD4 and CD8 surface antigens (25).

Recent evidence from several laboratories indicates that γ/δ^+ T cells play a major role in the immune response to mycobacteria. γ/δ^+ T cells specifically accumulated in draining lymph nodes when mice were immunized with *M. tb* (22). Furthermore, CD4⁻ CD8⁻ γ/δ^+ T cell clones with specificity for mycobacterial antigens have been isolated from patients suffering from rheumatoid arthritis (19), leprosy (21), and from normal PPD-immune individuals (20). Our present results extend these data to show that killed mycobacteria activate a large fraction of unselected γ/δ^+ T cells from normal, PPD-nonimmune individuals. A major question concerns the mycobacterial antigen(s) involved in stimulation of γ/δ^+ T cells. In the studies of Haregewoin et al. (20), γ/δ^+ T cell clones with MHC-restricted specificity for hsp 65 were derived from a Bacillus Calmette Guerin-immune individual after initial culture of T cells with PPD. Similarly, recombinant hsp 65 was found to stimulate certain murine γ/δ^+ T cell hybridomas that also responded to PPD in the absence of additional APC (23). In contrast, however, γ/δ^+ T cells isolated from granulomatous skin lesions in leprosy responded to *M. leprae* and PPD but not to hsp 65 (21). γ/δ^+ clones derived from rheumatoid synovium by Holoshitz et al. (19) specifically recognized an acetone-precipitable fraction of *M. tb* in an HLA class II-dependent but nonrestricted fashion; one of these clones responded to the biochemically purified hsp 64 from *M. bovis*.

Hsp are highly conserved, and a homologue of hsp 65 of *M. tb* has been identified in man that shares 65% sequence homology on the protein level (38). It has been proposed that γ/δ^+ T cells recognize autologous cells that have been stressed by invading microorganisms or other insults (18). Indeed, it has been shown that T cells (in this case, α/β^+) recognize stressed host cells (39), indicating that endogenous hsp can be presented in the context of MHC molecules. The observation that γ/δ^+ T cells respond to hsp 65 (19, 20), therefore, raises the question of whether these γ/δ^+ T cells recognize shared sequences of the bacterial and human hsp 65, and

primarily function as scavenger cells that are directed against host-derived hps. However, while such a mechanism of molecular mimicry may be involved in the immune response to mycobacteria (19–21), the results of our LD analyses suggest that hsp 65 stimulates only a minor fraction of γ/δ^+ cells from normal (PPD-nonimmune) donors (Fig. 1). A much higher fraction of peripheral blood γ/δ^+ T cells was activated by preparations of whole mycobacteria. The apparent specificity of many γ/δ^+ T cells for mycobacteria was maintained at the clonal level. We have established several γ/δ^+ clones from two healthy donors that specifically proliferated in response to mycobacteria but not in response to PPD or hsp 65. Restimulation of these clones required the presence of irradiated PBMC feeder cells; neither autologous EBV-transformed LCL nor MOLT-4 cells provided a suitable APC function. Based on the limited number of HLA-different PBMC tested here (Fig. 3), we did not find evidence for MHC class II restriction of these clones. In this regard, the clones described here appear similar to those established by Holoshitz et al. (19), and to γ/δ^+ lines derived from mice immunized with *M. tb* (22). It is not excluded, however, that mycobacteria-reactive γ/δ^+ clones are restricted by or dependent on HLA class I or class I-like molecules such as CD1 (11).

What is (are) the component of mycobacteria that activates γ/δ^+ T cells in such an efficient manner? Our results would argue that hsp 65 is not the major γ/δ^+ T cell-stimulating antigen of *M. tb* (at least for PPD-nonimmune individuals), although hsp 65 is recognized by some γ/δ^+ T cells. A variety of mycobacterial polysaccharides, proteins, and lipids have to be taken into consideration. Since we used only whole bacteria (apart from PPD and hsp 65), our present results do not allow us to further dissect the potential antigenic components. The potent stimulatory activity of mycobacteria on γ/δ^+ T cells is reminiscent of the recently described staphylococcal enterotoxin "superantigens" that stimulate a large fraction of α/β^+ T cells, presumably by directly crosslinking TCR and MHC class II molecules (40–42). However, such a mechanism does not appear to apply to mycobacteria-reactive γ/δ^+ T cells, since HLA class II-positive EBV-transformed LCL feeder cells failed to present mycobacteria to γ/δ^+ clones.

In summary, our studies have shown that *M. tb* organisms activate a large fraction of peripheral blood γ/δ^+ T cells. These results support the idea that γ/δ^+ cells play a major role in the defense against invading microorganisms. It remains to be investigated which components of mycobacteria are the major ligands for γ/δ^+ T cells.

Summary

We report that *M. tuberculosis* organisms, but neither PHA nor allogeneic stimulator cells, preferentially activate γ/δ^+ cells within E rosette-purified peripheral blood T cells. γ/δ^+ T cells from purified protein derivative (PPD)-nonimmune healthy donors were enriched by depletion of CD4⁺ and CD8⁺ cells; double-negative (DN) cells contained 65–92% γ/δ^+ T cells. Limiting dilution (LD) analyses revealed that 1 of 2–19 purified DN cells proliferated in response to mycobacteria, while frequencies of DN cells proliferating in response to a recombinant 65-kD heat shock protein (hsp 65) of *M. tuberculosis/M. bovis* were 10–20-fold lower. Established clones of mycobacteria-reactive γ/δ^+ T cells specifically recognized mycobacteria, but neither PPD nor hsp 65. Restimulation of these clones required the presence of PBMC feeder cells; EBV-transformed lymphoblastoid cell lines could not

substitute for PBMC. Mycobacteria-reactive γ/δ^+ clones proliferated equally well in the presence of autologous or allogeneic (HLA-DR-different) PBMC feeder cells and thus were not MHC class II restricted. Taken together, these results demonstrate that mycobacteria-reactive γ/δ^+ T cells are present in high frequency in the peripheral blood of healthy individuals, and suggest that hsp 65 of mycobacteria is not a major antigen for γ/δ^+ T cells of normal PPD-nonimmune blood donors.

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