Clin exp. Immunol. (1992) **89**, 301–304

Preferential activation of peripheral blood Vγ9$^+$ γ/δ T cells by group A, B and C but not group D or F streptococci

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(Accepted for publication 20 March 1992)

SUMMARY

Previous studies have established that inactivated mycobacteria are potent and selective activators of Vγ9$^+$/Vδ2$^+$ human γ/δ T cells. Here we have analysed the proliferative response of human γ/δ T cells to five serologically distinct groups of streptococci. While heat-inactivated streptococci of all five serogroups tested (A, B, C, D and F) induced a strong proliferative response in peripheral blood mononuclear cells (PBMC), only groups A, B and C elicited a selective activation of Vγ9$^+$ γ/δ T cells in 10 (serogroup B) or 11 (serogroups A and C) of 11 tested healthy individuals. In striking contrast, groups D and F streptococci failed to activate γ/δ T cells in nine of 11 donors and induced only a weak γ/δ T cell response in two additional individuals. Depletion of Vγ9$^+$ T cells before culture completely eliminated all γ/δ T cell responses to streptococci. These data indicate that groups A, B and C (but not D or F) streptococci can be included in the growing list of selective ligands for Vγ9$^+$/Vδ2$^+$ human γ/δ T cells.

Keywords γδ$^+$ T cell reactivity streptococci

INTRODUCTION

Several seemingly unrelated ligands including mycobacteria [1–4], Daudi [5–7] and MOLT-4 [4] tumour cells, *Plasmodium falciparum* merozoites [8], and staphylococcus enterotoxin A [9] have all been found to activate selectively Vγ9/Vδ2-expressing human γ/δ T cells. Apart from mycobacteria, other intracellular and extracellular bacteria reportedly stimulate human γ/δ T cells [10,11]; in these instances, however, the responsive γ/δ T cell subset(s) have not been identified in great detail. There is a striking bias towards Vγ9$^+$/Vδ2$^+$ cells among peripheral blood γ/δ T cells, in contrast to post-natal human thymus where such cells constitute only a minor fraction of γ/δ thymocytes [12,13]. It is postulated that the relative expansion of Vγ9$^+$/Vδ2$^+$ during childhood might be related to some sort of antigenic pressure elicited by, for example, exposure to bacterial antigens. In the present study we have analysed the activation of peripheral blood γ/δ T cells by serologically distinct groups of heat-killed streptococci. Our results indicate that groups A, B and C streptococci induce a selective proliferative response of Vγ9$^+$/Vδ2$^+$ γ/δ T cells in most healthy adult blood donors.

MATERIALS AND METHODS

Isolation of lymphocyte subsets

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood by Ficoll–Hypaque density gradient centrifugation. PBMC were depleted of Vγ9$^+$γ/δ T cells by sorting on a FACStar Plus cell sorter (Becton Dickinson, Heidelberg, Germany). To this end, PBMC were stained with biotinylated anti-Vγ9 MoAb 7A5 [14] followed by PE-labelled streptavidin (Becton Dickinson). Based on forward light scatter and fluorescence intensity, 7A5$^+$ cells were removed by cell sorting.

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Cell cultures

PBMC or Vγ9-depleted PBMC were cultured in triplicate at 1×10^5 cells/well in 96-well round-bottomed microtitre plates (Nunc, Wiesbaden, Germany). Culture medium was RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, antibiotics, and 10% heat-inactivated male human serum. Cultures were stimulated with 0·5 μg/ml PHA-P (Wellcome, Burgwedel, Germany) or an optimal concentration (0·001% v/v) of heat-killed bacteria. The following bacteria were employed: *Mycobacterium tuberculosis* strain H37Rv [1] or *Streptococci* serogroups A, B, C, D and F. All bacteria were kindly provided by Prof. H. G. Sonntag and Mrs R. Restorff (Institute of Medical Microbiology, University of Heidelberg, Germany). Bacteria were taken from agar plates, resuspended and washed three times in Dulbecco's PBS before being autoclaved for 20 min at 120°C. Subsequently, a 10% (v/v) stock dilution was stored at −70°C until use. After thawing, bacteria were sonicated for 5 min before use. All cultures were incubated for 3–8 days at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell proliferation was measured by uptake of ^{3}H-thymidine (^{3}H-TdR). To this end, cultures were pulsed with

Table 1. Kinetic of proliferative response of peripheral blood mononuclear cells (PBMC) to killed streptococci

	^{3}H-thymidine (mean ct/min of triplicate cultures $\times 10^3$)								
	Exp.1			Exp.2			Exp.3		
	day 3	day 6	day 8	day 3	day 6	day 8	day 3	day 6	day 8
—	0·3	2·4	1·7	0·3	0·3	2·3	0·3	4·4	5·1
PHA	169·7	25·0	21·5	115·6	23·1	4·8	109·2	23·8	11·4
M.tb.	1·4	39·3	11·1	1·9	16·0	7·6	12·1	78·3	10·7
Strep A	2·8	81·7	19·6	2·3	64·7	34·7	3·8	44·4	7·4
Strep B	2·2	38·5	8·0	1·5	63·7	21·3	7·7	82·9	8·8
Strep C	3·2	54·4	12·0	2·8	76·1	30·1	8·6	123·8	13·1
Strep D	0·7	27·0	27·9	1·0	24·7	26·2	3·5	56·0	4·5
Strep F	0·3	20·2	5·0	0·8	6·3	56·2	2·7	51·7	5·0

PHA, Phytohaemagglutinin; M.tb., *Mycobacterium tuberculosis*; strep, streptococci.

1 μCi ^{3}H-TdR/well (specific activity 6·7 Ci/mmole), and incubation was continued for 6 h. Cultures were harvested and prepared for counting of β emission in a Packard liquid scintillation counter. Results are expressed as mean ct/min of triplicate cultures. S.D. were always < 15% and, for the sake of clarity, have been omitted.

Phenotypic analysis of proliferating cells

The following MoAbs were employed for phenotypic analysis of T cell receptor (TcR) expression before and after culture: BMA031 (anti-TcR $\alpha\beta$; Behringwerke, Marburg, Germany; [15]); TCRδ1 (anti-Cδ; T Cell Sciences, Cambridge, MA; [16]); δTCS1 (anti-Vδ1/Jδ1 or Vδ1/Jδ2; T Cell Sciences; [17]); BB3 (anti-Vδ2; [18]); 7A5 (anti-Vγ9; [14]). TCRδ1 and δTCS1 were used as FITC conjugates. Biotinylated MoAbs BMA031 and 7A5 were visualized with streptavidin-PE as a second step reagent. FITC-conjugated F(ab')$_2$ goat anti-mouse IgG (Medac, Hamburg, Germany) was used to detect BB3$^+$ cells. All analyses were measured on a FACScan cytofluometer (Becton Dickinson).

RESULTS

Proliferative response of PBMC to killed streptococci

All five serogroups of streptococci (A, B, C, D, F) were found to stimulate a proliferative response in PBMC from normal adult blood donors. Dose response analyses revealed that a final concentration of 0·001% (v/v) was optimal for all bacteria (not shown). As illustrated in Table 1, ^{3}H-TdR incorporation following stimulation by heat-killed streptococci usually peaked on day 6 of culture, although a slightly delayed (day 8) optimum was noted for streptococci D and F in some experiments (e.g. Exp. 2, Table 1).

Phenotypic analysis of proliferating cells

Cytofluometric analysis of proliferating cells was performed after 6–9 days of culture to determine the TcR phenotype of T cells responding to heat-killed streptococci. Results of a representative experiment are depicted in Fig. 1. As can be seen, there was a dramatic increase of γ/δ T cells after stimulation with

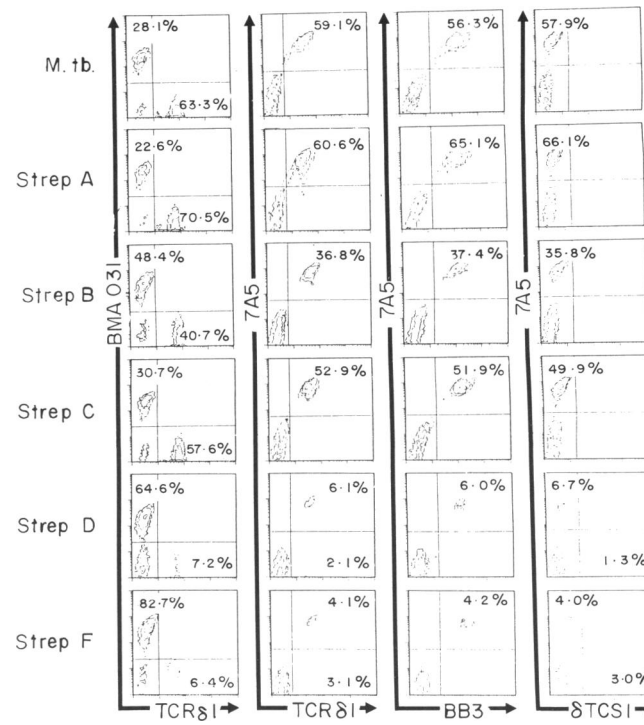

Fig. 1. Cytofluometric analysis of T cell receptor (TcR) expression after culture. Peripheral blood mononuclear cells (PBMC) were cocultured with the indicated killed bacteria. After 6–9 days, proliferating cells were double-stained with the indicated MoAbs and analysed on a FACScan cytofluometer. Abscissa: log green fluorescence (FITC); ordinate: log red fluorescence (PE). M.tb., *Mycobacterium tuberculosis*; strep, streptococcus.

Mycobacterium tuberculosis (M.tb.) and streptococci A, B and C, but not after stimulation with streptococci D or F. While TCRδ1$^+$ γ/δ T cells accounted for 4·6% of PBMC before culture, the percentage of TCRδ1$^+$ cells increased to range between 40·7 (streptococcus B) and 70·5% (streptococcus A) after culture. The detailed analysis with MoAbs specific for defined Vγ and Vδ gene products revealed that γ/δ T cells proliferating in response to M.tb. and streptococci A, B and C uniformly expressed Vγ9 (7A5) together with Vδ2 (BB3). In contrast, no Vδ1$^+$ (δTCS1$^+$) γ/δ T cells were detected after culture (Fig. 1). These results suggested that the proliferative response of γ/δ T cells to streptococci is restricted to Vγ9-bearing cells, similarly to what has been reported for M.tb. stimulation [2,4]. To prove this point, Vγ9$^+$ T cells were depleted before culture by removing 7A5$^+$ cells on a cell sorter. In this experiment, freshly isolated PBMC contained 6·7% TCRδ1$^+$ cells, of which 4·5% were 7A5$^+$/δTCS1$^-$ (i.e. Vγ9$^+$/Vδ1$^-$) and 2·3% 7A5$^-$/δTCS1$^+$ (i.e. Vγ9$^-$/Vδ1$^+$). As shown in Fig. 2, M.tb. and streptococcus A bacteria elicited a strong γ/δ T cell response in unseparated PBMC, thus giving rise to 80% γ/δ T cells after culture. In striking contrast, the γ/δ T cell response was completely eliminated in 7A5-depleted PBMC responder cells, indicating that activation of human γ/δ T cells by streptococcus A (and M.tb.; see [2]) is restricted to Vγ9-expressing cells.

Additional experiments were performed to address the issue of whether activation of γ/δ T cells by streptococci is exerted by some (A, B, C) but not other (D, F) serogroups. PBMC from 11

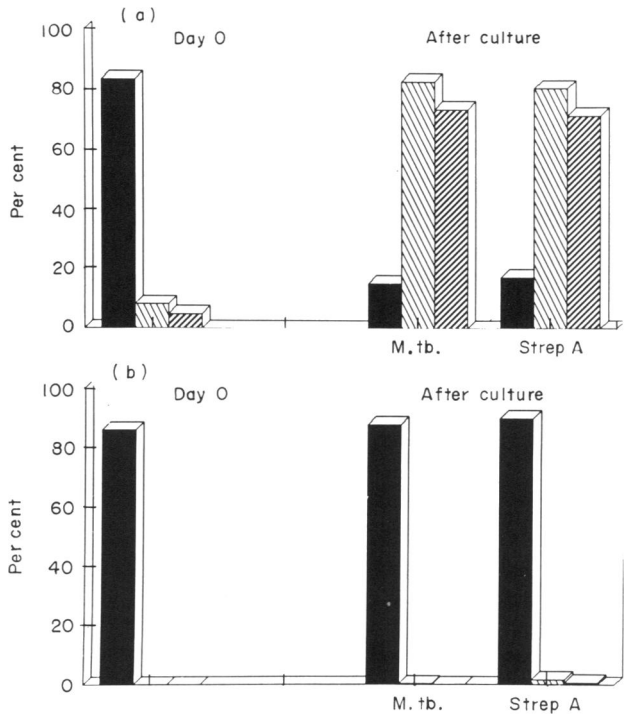

Fig. 2. Depletion of Vγ9⁺ T cells before culture eliminates all γ/δ T cell responses to bacteria. Peripheral blood mononuclear cells (PBMC) (a) and cell sorter Vγ9 (7A5)-depleted PBMC (b) were cocultured with *Mycobacterium tuberculosis* (M.tb.) or streptococcus A. After 8 days, proliferating cells were stained with MoAbs BMA031 (TcR αβ), TCRδ1 (Cδ), and 7A5 (Vγ9). The percentage of positive cells is indicated. ■, BMA031; ▧, TCRδ1; ■, 7A5.

randomly selected healthy adult individuals were stimulated with groups A, B, C, D and F streptococci, and the percentage of γ/δ (TCRδ1⁺) and Vγ9⁺ (7A5⁺) cells was determined before and after culture. As detailed in Table 2, streptococci A and C reproducibly elicited a strong γ/δ T cell response in all individuals tested, while streptococcus B was a potent γ/δ T cell inducer in 10/11 individuals. In contrast, both groups D and F streptococci failed to selectively activate γ/δ T cells in 9/11 individuals and induced only a weak γ/δ T cell response in the remaining two donors (Table 2). Note that in every single case the streptococci-reactive γ/δ T cells expressed Vγ9 (identified by MoAb 7A5), and not Vδ1 (identified by MoAb δTCS1). Vγ9⁺ T cells activated by group A, B or C streptococci displayed strong cytolytic activity towards bacteria-pulsed and non-pulsed autologous plastic-adherent cells (monocytes). However, in contrast to a previous publication [11], we were unable to discern a specific pattern of cytotoxicity in the sense that streptococcus A-activated γδ⁺ T cells would kill streptococcus A-pulsed but not streptococcus B (or mycobacteria)-pulsed monocytes (not shown).

DISCUSSION

The present results indicate that the capacity to activate Vγ9-expressing γ/δ T cells varies among serologically distinct subgroups of streptococci. It is intriguing to note that group D streptococci which are commonly present in the normal human intestine are poor activators of γ/δ T cells, while the pathogenic groups A, B and C streptococci elicit strong γ/δ T cell responses. Further studies are required to establish whether the potent *in vitro* reactivity of Vγ9⁺ T cells to groups A, B and C streptococci is based on previous *in vivo* exposure to these (or other) bacteria. We are currently analysing the N region diversity of streptococcus A-reactive Vγ9⁺ T cells to investigate whether the γ/δ T cell response is mono-, oligo-, or polyclonal in nature. A similar analysis of M.tb.-reactive Vγ9⁺ T cells revealed a broad N region diversity, indicating that M.tb. contains ligands which would qualify as superantigens for Vγ9⁺ γ/δ T cells [4]. If a comparable level of N region diversity is seen among streptococcus A-reactive Vγ9⁺ T cells, this might indicate that streptococcus A (but not D or F) similarly contains superantigen-like ligands for Vγ9⁺ γ/δ T cells. Indeed, our preliminary sequence analysis data of a limited number of streptococcus A-reactive Vγ9⁺ clones indicates a broad N region diversity (not shown).

There is a growing list of seemingly unrelated agents including bacteria, parasites and tumour cells which all activate

Table 2. T cell receptor (TcR) phenotype of T cells before and after culture with bacteria.

	day 0	Strep A		Strep B		Strep C		Strep D		Strep F		M.tb.	
Exp.	TCRδ1⁺	TCRδ1⁺	7A5⁺	TCRδ1⁺	7A5⁺	TCRδ1⁺	7A5⁺	TCRδ1⁺	7A5⁺	TCRδ1⁺	7A5⁺	TCRδ1⁺	7A5⁺
1	4·5	35·2	37·1	40·1	43·8	43·9	45·9	2·2	1·4	1·9	1·3	14·8	13·9
2	2·6	18·5	17·4	2·5	1·7	26·5	25·4	4·1	2·9	2·0	1·5	ND	ND
3	4·8	45·2	44·4	14·0	17·7	35·1	32·2	0·3	0·5	2·8	3·2	62·1	59·2
4	3·3	53·6	54·8	23·4	27·0	34·3	36·2	1·9	2·3	1·1	0·8	73·6	73·9
5	7·5	19·9	22·8	54·4	51·9	62·0	60·9	14·2	13·4	21·7	21·8	57·0	54·6
6	4·2	26·4	26·0	16·8	15·1	20·7	20·4	5·1	3·8	5·8	4·2	26·9	25·4
7	3·6	27·5	25·5	18·6	15·9	42·7	41·9	3·0	2.3	3·3	2·3	10·9	10·1
8	4·1	58·4	58·3	39·2	39·1	64·3	64·3	0·9	0·8	0·8	0·5	37·2	37·0
9	1·7	21·8	21·0	22·5	22·5	40·1	33·8	1·4	1·4	0·6	0·6	27·5	29·0
10	6·4	49·2	49·7	26·7	22·1	48·8	48·6	16·3	15·5	9·6	9·2	ND	ND
11	2·6	68·8	60·6	40·5	36·8	57·2	52·9	7·4	6·1	7·9	4·1	61·5	59·1

Strep, streptococcus; M.tb., *Mycobacterium tuberculosis*.

the same subset (i.e. $V\gamma9^+/V\delta2^+$) of human γ/δ T cells. Given that $V\gamma9^+/V\delta2^+$ cells constitute a minor γ/δ T cell subset in cord blood and post-natal thymus, it is postulated that $V\gamma9^+/V\delta2^+$ cells expand post-natally in response to an as yet unidentified stimulus [12]. At present, however, it is uncertain whether any of the above mentioned $V\gamma9$-specific ligands plays a role in the *in vivo* selection of such cells during childhood [19].

ACKNOWLEDGMENTS

We would like to thank our colleagues Drs R. Kurrle and L. Moretta for antibodies, and Mrs Ann Atzberger for operating the cell sorter. This work received financial support from the Alfried Krupp Award for young professors (to D.K.), and is in partial fulfilment of A.B.'s PhD thesis.

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