

## **BCG induced CD4<sup>+</sup> cytotoxic T cells from BCG vaccinated healthy subjects: relation between cytotoxicity and suppression *in vitro***

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### SUMMARY

Mycobacterial antigen specific cytotoxic T cells killing antigen-pulsed antigen presenting cells (APC) were induced from peripheral blood mononuclear cells (PBMC) of BCG-vaccinated healthy subjects after activation *in vitro* with BCG. CD8<sup>+</sup> depleted cells were as effective as PBMC, indicating that CD4<sup>+</sup> cells play a dominant role in this phenomenon. CD4<sup>+</sup> T cell clones raised against BCG also exhibited mycobacterial antigen specific cytotoxicity and suppressed BCG-driven selfproliferation. However, the same clones could either suppress or enhance the proliferation of other T cell clones from the same subject. The possible function *in vivo* of the cytotoxicity mediated by CD4<sup>+</sup> T cells is discussed.

**Keywords** BCG antigen presenting cells cytotoxic cells CD4<sup>+</sup> T cell clones suppression

### INTRODUCTION

BCG activation of peripheral blood mononuclear cells from BCG-vaccinated healthy subjects has been shown to induce mycobacterial antigen specific suppressor T cells capable of suppressing antigen driven proliferation of fresh T cells (Mustafa & Godal, 1983). These suppressors were MHC restricted and had the CD4<sup>+</sup> phenotype. The studies on the mechanism of suppression revealed that it was not mediated by soluble factors. Interleukin 2 activity was reduced in the cultures with suppressor cells. However, exogenous addition of recombinant IL-2 could only partially abrogate the suppression. This could have been due to suppression of IL-2 receptor expression by fresh T cells (Mustafa & Godal, 1985).

Antigen on the surface of antigen presenting cells (APC) triggers primed T cells to produce IL-2 and to express IL-2 receptors, the two prerequisites for T cell proliferation (Cantrell & Smith, 1984; Waldmann, 1986). Cytotoxic T cells can downregulate their IL-2 receptor expression and IL-2 production by killing the antigen presenting cells. Reexpression of IL-2 receptors and IL-2 production requires fresh antigen on an appropriate APC (Wee & Bach, 1984; Pawlec *et al.*, 1984; Kaplan, Braciale & Braciale, 1984; Lowenthal *et al.*, 1985; Andrew *et al.*, 1985; Nakamura *et al.*, 1986). Therefore, we have investigated the possibility of induction of cytotoxic T cells which could suppress proliferation by killing antigen-pulsed APC. Our findings show that such cytotoxicity may contribute to T cell mediated suppression *in vitro*.

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## MATERIALS AND METHODS

*Reagents*

Ficoll-Hypaque was purchased from Nyco, Oslo, Norway. Tissue culture medium RPMI-1640 was obtained from Gibco, Europe. Pooled normal human AB serum was supplied locally by blood bank. The complete medium contained RPMI 1640 with 15% heat-inactivated AB serum and 1% penicillin-streptomycin.  $^3\text{H}$ -thymidine (specific activity:  $185 \times 10^3$  MBq/mmol) and  $\text{Na}_2$   $^{51}\text{CrO}_4$  were purchased from Institute for Energiteknikk, Kjeller, Norway.

Live BCG from liquid cultures and PPD were obtained from Statens Serum Institute, Copenhagen, Denmark. *Mycobacterium tuberculosis* H37Rv, killed by irradiation, was supplied by Dr O. Closs, Institute for Public Health, Oslo, Norway. Killed armadillo-derived *M. leprae* was obtained from Dr R. J. W. Rees through the IMMLEP (WHO) Bank and Streptokinase-streptodornase (SKSD) from Cynamid Iberica, SA, Madrid, Spain.

*Effector cells for cytotoxicity assay*

Human peripheral blood mononuclear cells (PBMC) were obtained from the heparinized blood of BCG-vaccinated healthy subjects by Ficoll-Hypaque (lymphoprep), density centrifugation. Peripheral blood mononuclear cells,  $20 \times 10^6$  suspended in 10 ml complete medium, were cultured with BCG (20  $\mu\text{g}/\text{ml}$ , wet weight) in 50 ml tissue culture flasks (Costar, Cambridge, Massachusetts, USA) as described earlier for suppressor cell induction (Mustafa & Godal, 1985). On day 8, viable cells were recovered on lymphoprep gradient, washed three times with RPMI-1640 and tested for their cytotoxic activity. To remove  $\text{CD8}^+$  cells, PBMC precultured with BCG were treated with OKT8 monoclonal antibody + complement (Mustafa & Godal, 1983).  $\text{CD4}^+$  T cell clones raised against BCG from BCG-vaccinated healthy subjects (Mustafa *et al.*, 1986) were also used as effectors in cytotoxicity assay.

*Cytotoxicity assays*

*$^{51}\text{Cr}$  release assay.* Fresh monocyte enriched adherent cells were obtained from  $30 \times 10^6$  PBMC in 50 ml flasks (Costar) as described earlier (Mustafa & Godal, 1985). To differentiate into macrophages, adherent cells were cultured *in vitro* for 7 days at  $37^\circ\text{C}$ . Monocyte-enriched adherent cells and differentiated macrophages were pulsed overnight with antigens in 3 ml complete medium. BCG was used at 200  $\mu\text{g}/\text{ml}$ , PPD at 20  $\mu\text{g}/\text{ml}$  and SKSD at 200 SK U/ml. The cells were released from plastic surface by incubating the flasks for 1 h on ice followed by vigorous pipetting, washed twice with RPMI 1640 and suspended in 1 ml complete medium in siliconized glass tubes. The cells were labelled with 100  $\mu\text{Ci}$  of  $\text{Na}_2$   $^{51}\text{CrO}_4$  by incubating the tubes for 1 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , and washed three times with RPMI-1640.

EBV-transformed B cell lines were raised from PBMC of BCG vaccinated subjects according to the method of Bird, McLachlan & Britton (1981). EBV-transformed B cells,  $5 \times 10^6$  suspended in 1 ml complete medium with or without 100  $\mu\text{g}$  PPD, were labelled with 100  $\mu\text{Ci}$  of  $\text{Na}_2$   $^{51}\text{CrO}_4$  (as described for adherent cells).

Aliquots of  $10^4$  labelled target cells in 0.1 ml complete medium were seeded into wells of 96-well microtitre plates (Costar, Cambridge, Massachusetts, USA). Effector cells in 0.1 ml complete medium were added in triplicates at effector to target cell ratios of 1:1, 10:1 and 100:1. The plates were centrifuged at 50 g for 5 min and incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 4 h. The plates were then centrifuged at 500 g for 5 min. One hundred microlitres of supernatant were removed from each well and transferred to tubes for counting in a gamma counter. Median values of ct/min have been used to calculate percentage cytotoxicity, which is defined as:

$$\text{cytotoxicity (\%)} = \frac{\text{ct/min experimental} - \text{ct/min spontaneous}}{\text{ct/min total} - \text{ct/min spontaneous}} \times 100$$

where 'ct/min total' is the radioactivity released from the target cells in the presence of 1% triton X-100, 'ct/min spontaneous' is the radioactivity released from target cells incubated with complete

medium alone, and 'ct/min experimental' is the radioactivity released in the presence of effector cells.

*Neutral red assay.* Fresh monocyte-enriched adherent cells or 1-week-old macrophages differentiated *in vitro* from  $1 \times 10^6$  PBMC in 24-well plates (Costar) were pulsed with antigens. BCG-induced CD4<sup>+</sup> T cell clones were added, and the plates were incubated either overnight or for 6 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cultures were subsequently washed with warm RPMI 1640. The remaining adherent cells were incubated for 1 h at 37°C with a 1-week-old solution of 0.04% neutral red in RPMI 1640 + 10% FBS, and, thereafter, washed twice with warm saline + 10% FBS and once with saline alone. A solution of 0.05 M acetic acid in 50% ethanol, 0.5 ml, was added into each well to release neutral red from adherent cells (Parish & Müllbacher, 1983). OD<sub>540</sub> was determined on a spectrophotometer and percentage cytotoxicity was calculated from the following formula:

$$\text{cytotoxicity (\%)} = \frac{\text{OD}_{540} \text{ control} - \text{OD}_{540} \text{ experimental}}{\text{OD}_{540} \text{ control}} \times 100$$

where OD<sub>540</sub> control = OD<sub>540</sub> of cultures with adherent cells + antigen, OD<sub>540</sub> experimental = OD<sub>540</sub> of cultures with adherent cells + T cell clone + antigen.

#### *Suppressor assay*

One hundred thousand PBMC, irradiated with 2,500 rad, in complete medium were added to each well of 96-well flat-bottomed microtitre plates and incubated for 1 h in an atmosphere of 5% CO<sub>2</sub> and 95% air. Nonadherent cells were washed and adherent cells were used as APC. Ten thousand T cell clones were added to the wells and stimulated with BCG. To determine the suppressor effect of T cell clones,  $1 \times 10^5$  irradiated cloned cells were added to the wells in triplicate. The total culture volume was kept at 200 µl. The plates were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Four hours before harvesting, cultures were pulsed with 0.05 MBq <sup>3</sup>H-thymidine. The cultures were harvested and the radioactivity incorporated was measured by standard procedures (Mustafa & Godal, 1983). The median ct/min from triplicates were used to express results and to calculate percentage suppression which is defined as:

$$\text{Suppression (\%)} = 1 - \frac{\text{ct/min of cultures with } 1 \times 10^4 \text{ cloned T cells + APC + BCG + } 1 \times 10^5 \text{ irradiated cloned T cells}}{\text{ct/min of cultures with } 1 \times 10^4 \text{ cloned T cells + APC + BCG}} \times 100.$$

## RESULTS

*Cytotoxicity mediated by PBMC precultured with BCG.* PBMC cultures with BCG for induction of suppressor T cells (Mustafa & Godal, 1983), when seen under an inverted microscope were devoid of adherent macrophages, whereas cultures without BCG had a confluent monolayer of differentiated macrophages. The absence of a macrophage monolayer in cultures with BCG was not due to their detachment from the surface, as <0.1% esterase positive cells were found in the non-adherent cell population. Another possibility could have been the induction of cytotoxic cells killing monocytes/macrophages in the presence of BCG. Experiments done to substantiate it in quantitative terms using <sup>51</sup>Cr release assay showed that BCG and PPD pulsed monocyte enriched adherent cells were specifically killed by PBMC precultured with BCG. Killing was greatest at effector to target cell ratio of 100:1 and decreased at lower effector cell numbers (Table 1). The killing of monocyte-enriched adherent cells required specific antigen as targets with no antigen pulsing or pulsed with SK-SD, were not killed (Table 1). Similarly control PBMC cultures that did not have BCG during induction phase or cell free supernatants from BCG containing cultures could not kill BCG pulsed or non-pulsed adherent cells (data not shown). Antigen pulsed macrophages differentiated *in vitro* and EBV-transformed B cell lines were also killed by BCG-activated cytotoxic

**Table 1.** Cytotoxicity of BCG precultured PBMC for antigen pulsed monocyte enriched adherent cell targets (mean percentage cytotoxicity\*  $\pm$  s.e.m.)

Antigens for target pulsing	E:T		
	100:1	10:1	1:1
No antigen pulsing ( $n=9$ )	9.0 $\pm$ 2.4	2.5 $\pm$ 2.1	1.0 $\pm$ 1.0
BCG ( $n=4$ )	43.0 $\pm$ 11.8	27.2 $\pm$ 7.2	8.3 $\pm$ 4.8
PPD ( $n=9$ )	37.4 $\pm$ 6.4	25.0 $\pm$ 8.4	7.5 $\pm$ 2.5
SKSD ( $n=2$ )	11.5 $\pm$ 4.4	NT	NT

  

Targets	Mean percentage spontaneous $^{51}\text{Cr}$ release $\pm$ s.e.m.
Antigen nonpulsed monocytes	23.0 $\pm$ 4.0
BCG pulsed monocytes	27.7 $\pm$ 2.1
PPD pulsed monocytes	23.7 $\pm$ 3.9
SKSD pulsed monocytes	26.0 $\pm$ 0.7

\* Assessed by  $^{51}\text{Cr}$  release assay.

$n$ , No. of experiments.

NT, not tested.

**Table 2.** Cytotoxicity of BCG-activated PBMC against EBV transformed autologous B cell lines pulsed with PPD

No. of experiments*	Target pulsed with	Effector to target cell ratio (%) cytotoxicity	
		1:1	10:1
1	No antigen	4	14
	PPD	9	39
2	No antigen	—	0
	PPD	9	33
3	No antigen	0	13
	PPD	3	34
Mean cytotoxicity	No antigen	1	9
	PPD	7	35
Mean specific cytotoxicity		6	26

\* The experiments were done using BCG activated PBMC and EBV transformed autologous B cell lines from three different donors.

**Table 3.** Specificity of cytotoxicity of BCG induced CD4<sup>+</sup> T cell clones against autologous monocyte enriched adherent cells in the presence of different antigens (% cytotoxicity)

CD4 <sup>+</sup> T cell clones	Antigens			
	BCG	<i>M. tuberculosis</i> H37Rv	<i>M. leprae</i>	SK-SD
(1) 130/83	100	100	-30	-13
(1) BC-10	100	100	-8	-11
(2) BC-10	97	98	87	14
(3) BC-10	97	98	0	2
(5) BC-10	94	96	7	-5

1 × 10<sup>6</sup> irradiated T cell clones were added into each well of 24 well Costar plates with autologous adherent cells from 1 × 10<sup>6</sup> irradiated PBMC. BCG and *M. tuberculosis* were added at 20 µg/ml (wet weight), *M. leprae* at 5 × 10<sup>7</sup> bacilli/ml and SKSD at 50 SK U/ml. Neutral red uptake was assessed on day 6 of incubation. % cytotoxicity was calculated as described in Materials and Methods.

**Table 4.** Dose dependent cytotoxicity of BCG-induced CD4<sup>+</sup> T cell clones in the presence of BCG against autologous macrophages differentiated *in vitro*

No. of cloned cells/well	Cytotoxicity at			
	18 h		6 days	
	OD <sub>540</sub>	% cytotoxicity	OD <sub>540</sub>	% cytotoxicity
None	0.27	—	0.33	—
1 × 10 <sup>6</sup>	0.07	74	0.00	100
1 × 10 <sup>5</sup>	0.25	7.4	0.00	100
1 × 10 <sup>4</sup>	0.34	-26	0.14	57
1 × 10 <sup>6</sup> irradiated	0.1	63	0.00	100
1 × 10 <sup>5</sup> irradiated	0.28	-4	0.12	64
1 × 10 <sup>4</sup> irradiated	0.34	-26	0.28	15

Data are presented for clone (1) 130/83. Similar results were obtained with clones of BC-10.

cells. In an experiment, 38% and 42% cytotoxicity was found for BCG and PPD pulsed 7-day-old macrophages differentiated *in vitro* and 26% mean specific cytotoxicity was observed at an effector to target cell ratio of 10:1 using PPD pulsed EBV transformed B cell lines as targets (Table 2). PBMC precultured with BCG and depleted of CD8<sup>+</sup> cells were as potent effectors (% cytotoxicity = 37%) for BCG-pulsed monocyte enriched adherent cells as total PBMC (% cytotoxicity = 34%).

*Cytotoxicity mediated by CD4<sup>+</sup> T cell clones.* One hundred and twenty CD4<sup>+</sup> T cell clones were raised from nine BCG vaccinated healthy subjects (Mustafa *et al.*, 1986). As expected, all of these clones periodically required, in addition to interleukin 2 and BCG, adherent APC for their continuous growth *in vitro*. However, after 10–14 days of adherent cell feeding, the clones ceased to

**Table 5.** Effect of CD4<sup>+</sup> T cell clones on BCG induced selfproliferation and on the proliferation of other clones from the same subject

$1 \times 10^5$ irradiated cells added from	BCG induced proliferation (ct/min) of clones				
	(1) 130/83	(1) BC-10	(2) BC-10	(3) BC-10	(5) BC-10
None	9566	16078	34266	4032	6333
(1) 130/83	5097 (47)	NT	NT	NT	NT
(1) BC-10	NT	4141 (74)	10300 (70)	12694 (-215)	9025 (-42)
(2) BC-10	NT	15528 (3)	10950 (68)	25011 (-520)	16200 (-155)
(3) BC-10	NT	879 (94)	630 (98)	727 (82)	951 (85)
(5) BC-10	NT	865 (95)	1213 (96)	2134 (47)	1680 (73)

Values in parentheses represent percentage suppression.  
NT, not tested.

grow even in the presence of IL-2 and the presence of antigen and absence of adherent cells on microscopic examination of cultures suggested that the clones might have killed adherent APC in the presence of BCG. The observation was quantitatively substantiated by neutral red uptake by the remaining adherent cells. The killing was antigen-specific as all the five clones tested killed adherent cells in presence of BCG and *M. tuberculosis* H37Rv; only one clone, (2) BC-10, could kill adherent cells in the presence of *M. leprae* and none killed adherent cells in the presence of SK-SD (Table 3). The T cell clones in the presence of BCG were also cytotoxic for 7-day-old macrophages differentiated *in vitro*. The clones at  $1 \times 10^6$  cells/well and  $1 \times 10^5$  cells/well killed macrophages by day 6. At lower effector cell numbers, non-irradiated clones were better killers than irradiated clones. Cytotoxicity after overnight incubation was observed only at  $1 \times 10^6$  cloned cells/well (Table 4).

*Suppression mediated by CD4<sup>+</sup> T cell clones.* All of the five clones suppressed their own BCG induced proliferation when  $1 \times 10^5$  irradiated cells were added to  $10^4$  non-irradiated responding cells in 96-well plates (Table 5). However, with respect to the effect on the proliferation of other clones from the same subject, (1) BC-10 and (2) BC-10, at selfsuppressive concentration, enhanced the proliferation of (3) BC-10 and (5) BC-10, whereas, (3) BC-10 and (5) BC-10 were suppressive for all four clones (Table 5).

## DISCUSSION

In this study we have demonstrated induction *in vitro* of cytotoxic cells from PBMC of BCG-vaccinated healthy subjects capable of killing BCG pulsed monocyte enriched adherent cells. As the cells of monocyte/macrophage lineage could be the major cells for presentation to T cells of complex bacterial antigens (Unanue *et al.*, 1984), killing of such cells could result in the suppression of T cell proliferation. Results of this study show that induction of cytotoxicity from PBMC paralleled closely the induction of the T cell-mediated suppression described earlier (Mustafa & Godal, 1983; 1985). For example, suppressor and cytotoxic cells were induced under identical culture conditions, were antigen specific, depletion of CD8<sup>+</sup> cells did not abrogate their activity, and their effect was not mediated by cell free supernatants, etc.

Mycobacterial antigen specific CD4<sup>+</sup> T cell clones from BCG vaccinated healthy subjects (Mustafa *et al.*, 1986) were either specific to BCG and *M. tuberculosis* e.g. (1) 130/83 or limited to broadly crossreactive, e.g. clones of BC-10, in proliferative assays. These clones were producing multiple lymphokines like IL-2,  $\gamma$ -interferon and granulocyte/macrophage colony stimulating factor activity (Mustafa *et al.*, 1986). When tested for cytotoxicity, these clones exhibited mycobacterial antigen specific killing of monocyte-enriched adherent cells (Table 3).  $\lambda$ -Repressor antigen specific, IL-2 producing T cell clones with L3T4<sup>+</sup>, LYt2<sup>-</sup> phenotype (Nakamura *et al.*, 1986) and *L. monocytogenes* raised,  $\gamma$ -interferon producing LYt2<sup>+</sup> T cell clones (Kaufmann, Hug & De Libero, 1986) have recently been shown to be cytotoxic for antigen-presenting cells. However, in humans, this is the first report, to our knowledge, of mycobacterial antigen specific, multiple lymphokine producing T cell clones of helper phenotype mediating antigen specific killing of APC.

A number of investigators have successfully used the cells of monocyte/macrophage lineage as targets of cytotoxic T cells either using <sup>51</sup>Cr release assay (Parish & Müllbacher, 1983; Kaufmann *et al.*, 1986; De Libero & Kaufmann, 1986) or neutral red uptake assay (Parish & Müllbacher, 1983; Müllbacher, Parish & Mundy, 1984; Chiplunkar *et al.*, 1986; De Libero & Kaufmann, 1986). Uptake of neutral red by remaining viable adherent cells to measure the cytotoxicity of T cells against thioglycollate-induced mouse macrophages was developed by Parish and Müllbacher. The method is cheap, reliable, more sensitive than <sup>51</sup>Cr release assay, avoids the hazards of radioactivity and can be safely used even after prolonged periods of effector and target cell interaction (Parish & Müllbacher, 1983; Müllbacher *et al.*, 1984). Our results show that neutral red uptake can also be used to quantify the killing of human macrophages differentiated *in vitro* in overnight as well as day 6 assays. However, killing of fresh monocytes by T cells could not be quantified by dye uptake in overnight assays because fresh monocytes were poor in retaining the dye during washing steps. Therefore, the cytotoxic effect of T cell clones on fresh adherent cells was assessed after 6 days of incubation by which time monocytes had differentiated into macrophages. Assaying cytotoxicity on day 6 minimizes non-adherence of macrophages to the plastic surface which may happen with fresh monocytes due to endocytosis and clustering during early phases after antigen pulsing. The T cell clones in the presence of antigen and APC show optimal proliferative response around day 2–3, and in the absence of exogenous IL-2, most of the cells at  $1 \times 10^6$ /well are dead by day 6. Thus, nonadherence of macrophages to the plastic due to vigorous lymphocyte-macrophage interaction was also avoided. The enhancement of dye uptake by macrophages due to their activation by T cells, either directly or via lymphokines, would be a problem with neutral red assay (Müllbacher *et al.*, 1984). This may be the reason for negative percentage cytotoxicity at  $1 \times 10^4$  cloned cells/well in Table 4.

CD4<sup>+</sup> cytotoxic T cell clones can also have suppressor activity, as is suggested from the experiments where they suppressed BCG-induced selfproliferation. However, variable effects were observed on the proliferation of other clones from the same subject. For example, selfsuppressive clones (3) BC-10 and (5) BC-10 suppressed the proliferation of other clones as well, whereas selfsuppressive clone (2) BC-10 enhanced the proliferation of other clones (Table 4). Kotani *et al.* (1986) and Champion *et al.* (1986) have also reported T cell clones with helper phenotype that depending upon the responding cells either helped or suppressed immunoglobulin production.

What role might mycobacteria specific CD4<sup>+</sup> cytotoxic T cells have *in vivo*? Since mycobacteria are killed intracellularly, one would at first sight think that such cells would be deleterious. However, it is well known that mycobacteria are difficult for macrophages to deal with. Probably they only have a short span in which to exert their antibacterial effects. Thus the only way by which the body may attack bacteria inside older macrophages may be by first lysing them and so exposing the bacteria to younger invading monocytes. This could also dilute the number of bacteria per cell.

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