SHORT COMMUNICATION

Kinetics of T Cell-activation Molecules in Response to *Mycobacterium tuberculosis* Antigens

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The phenotypic features acquired subsequent to antigen-specific stimulation in vitro were evaluated by means of the kinetic expressions of CD69 and CD25 activation molecules on T lymphocytes and assayed by flow cytometry in response to PPD, Ag85B, and ferritin in PPD-positive healthy control individuals. In response to PHA, CD69 staining on both CD4+ and CD8+ T cells became initially marked after 4 h, peaked at 24 h, and quickly decreased after 120 h. For CD25, a latter expression was detected around 8 h, having increased after 96 h. As expected, the response rate to the mycobacterial antigens was much lower than that to the mitogen. Positive staining was high after 96 h for CD25 and after 24 h for CD69. CD69 expression was significantly enhanced ($p < 0.05$*) on CD8+ as compared to CD4+ T cells. High levels were also found between 96-120 h. Regarding Ag85B, CD25+ cells were mostly CD4+ instead of CD8+ T cells. Moreover, in response to ferritin, a lower CD25 expression was noted. The present data will allow further characterization of the immune response to new mycobacterial-specific antigens and their evaluation for possible inclusion in developing new diagnostic techniques for tuberculosis as well in a new vaccine to prevent the disease.*

Key words: activation markers - CD69 - CD25 - flow cytometry

An essential requirement for the effective control of tuberculosis (TB), a major health care problem worldwide, is the characterization of the *Mycobacterium tuberculosis* (Mtb) antigens (Ags) that are recognized by T cells. There has been a concentrated effort over the last few years to characterize specific Mtb molecules for inclusion in a novel TB vaccine and development of more efficacious diagnostic tools. Up to now, evaluations of the functional T cell response in vitro have been based on the secretion of cytokines (mainly IFNγ) and on the expression of activation molecules on the cell surface. Nevertheless, very little information on the phenotypic changes present in the antigen-specific stimulated cells has been made available (Hviid et al. 1993). Based on the recognition of Mtb Ags, the objective of the current study was to evaluate the kinetic expression of early activation molecules (CD69 and CD25) in response to PPD (Statens Serum Institute, Denmark) and the Mtb recombinant proteins Ag85B and ferritin (10 µg/ml) in primary peripheral blood mononuclear cell (PBMC) cultured in 96-well round-bottom plates at 200 µl of 2.0 x 10^5 cells/well. A total of 5 PPD-positive healthy control individuals (HC) from Rio de Janeiro were tested.

CD69 and CD25 (IL-2Rα) expression was assessed on T lymphocytes by means of flow cytometry in order to identify antigen-primed specific cells directly from the blood. The kinetic response for PHA (1%; Gibco Laboratories, Gaithersburg, MD) was also evaluated in culture at different time periods ranging from 2-120 h. The detection of activated T cells was assayed by using FITC- anti-hCD4 or CD8 plus PE- anti-hCD25 or CD69 in cold PBS plus 1% BSA. Labeled cells were analyzed on a FACScalibur® device (Becton & Dickinson Co., Mountain View, CA). A cell gate region was drawn around lymphocytes in order to exclude debris, and a total of 10,000 events per sample were collected. Thresholds and statistical markers were set for positivity by means of irrelevant isotype FITC- and PE-mIgG1 in match controls as a reference. All data were expressed as the percentage of double stained-positive bright cells.

When PBMC were stimulated in vitro with PHA, a significant expression of CD69 on both CD4+ (already present after 4 h stimulation) and CD8+ T cells (Fig. 1) was detected after 12 h (32.1% and 40.1%, respectively), reaching a peak after 24 h (39.6% and 42.6%). CD69 levels then proceeded to fast decrease, being much lower after 120 h (Fig. 1A). Staining for CD25 (Figs 1B, C) initially showed a latter expression around 8 h. After 12 h, 27.5% and 20.5% of the positive cells were detected on both

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CD4+ and CD8+ subsets, respectively. Peak of response in both populations occurred after 96 h (CD4+ = 44.6%; CD8+ = 41.4%), apparently having reached a plateau (Figs 1B, C). The present data are in accordance to those from Iannone et al. (1994). It is especially noteworthy that the CD25+ background levels (7.3%) observed in the non-stimulated cultures were only detected on CD4+ T cells (Fig. 1B).

A recent study reported that the analysis of CD25 expression on activated lymphocytes can be useful in measuring cellular immunity to PPD in goats (Storset et al. 2000). For this reason, PPD, Ag85B, and ferritin were also used herein for purposes of comparison (Table). Albeit the kinetic of response to the antigen was similar to that of the mitogen, the rate of positive cells detected in vitro was much lower (Table). CD69+ cells in the PPD stimulated cultures (CD4+ = 3.1%; CD8+ = 9.6%) were already largely enhanced after 12 h and were for the most part detected 24 h after stimulation (Figs 2A, B); even so, high levels of mainly CD8+/CD69 cells were also found after 96-120 h (Fig. 2B). With respect to CD25, positive staining (CD4+/CD25) maximized in the 96 h cultures (Fig. 2C). As observed in the Table, the CD25 rate in CD8+ T cells (96 h) was similar (CD4 = 7.2%; CD8 = 7%). Kinetic analysis showed the induction of CD25 expression by Ag85B to be also higher after 96 h of culture, particularly on CD4+ (4.7%) as opposed to CD8+ (2.3%; Table; Fig. 2A) T cells ($p < 0.01$). More interestingly, stimulation with ferritin revealed a much lower induction of CD25 in vitro (Table) on both T cell subsets. As regards CD69 at 24 h of culture, the same pattern of response was noted for all mycobacterial Ags whose expression was predominantly detected on CD8 as compared to CD4 T cells (Table).

In immune competent hosts, protective T cell immune responses are characterized by major effector functions, which are essential for controlling mycobacterial replica-

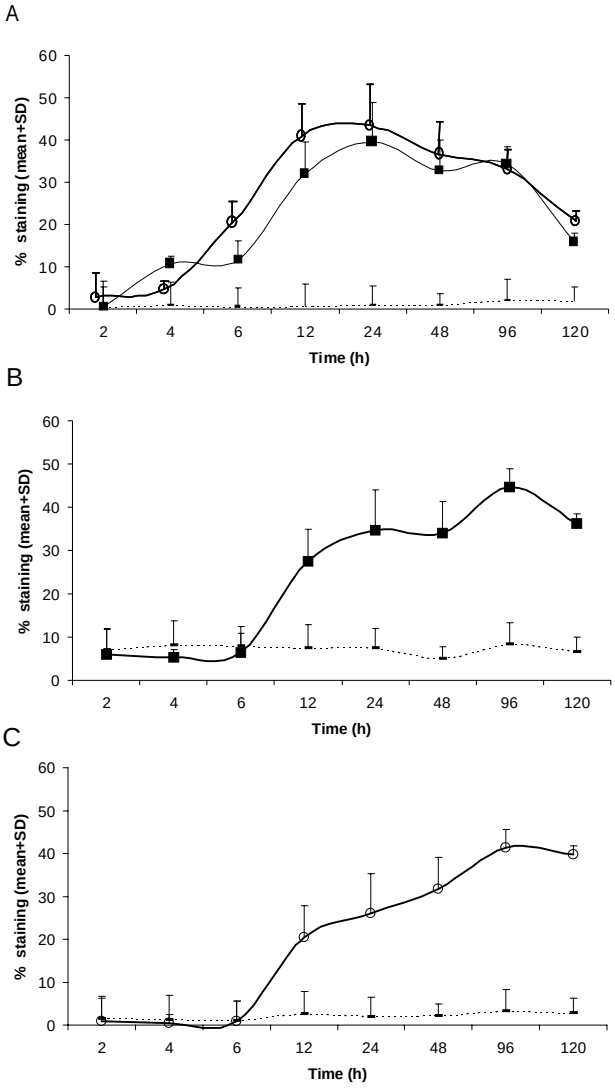

Fig. 1: time-point (mean + SEM) kinetic of (A) CD69 and (B-C) CD25 expression in Phytohemaglutinin-stimulated (A-B) CD4+ and (A-C) CD8+ T cells in vitro as evaluated by flow cytometry. Closed squares stand for CD4 and open circles for CD8. Dashed line represents the non-stimulated cultures.

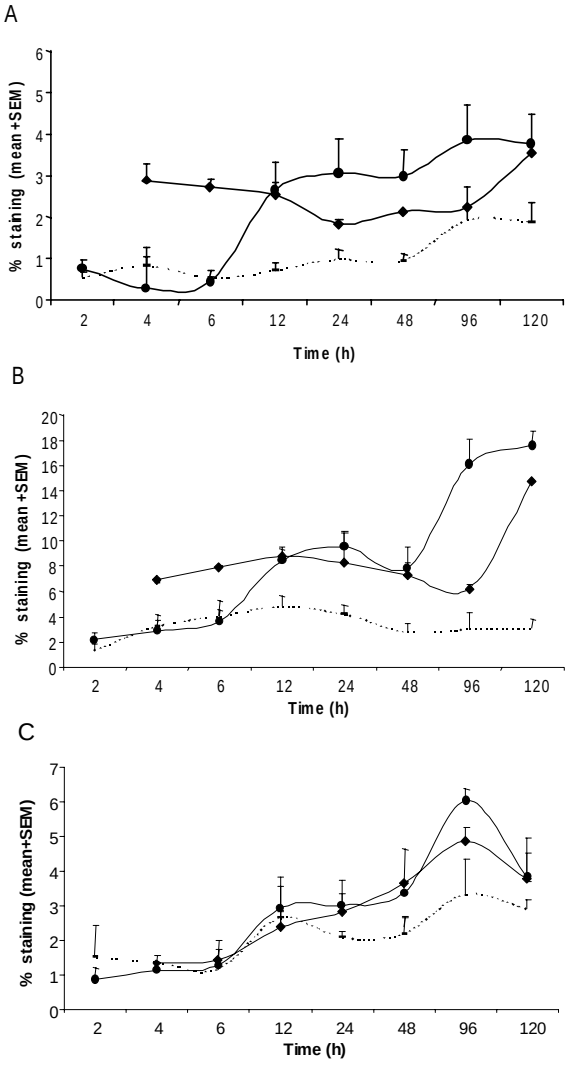

Fig. 2: time-point (mean + SEM) kinetic of (A) CD4+, (B) CD8+/CD69, and (C) CD4+/CD25 staining in the mononuclear cell cultures stimulated in vitro with purified protein derivative (closed circles) and antigen 85B (closed diamonds) as evaluated by flow cytometry. Dashed line represents the non-stimulated cultures.

TABLE

Percentage of T cells showing activation markers following in vitro antigen or mitogen stimulation

Activation markers	Stimulus [e]			
	PPD	Ag85B	Ferritin	PHA
CD4/CD25 [a]	7.2 ± 1.5 [b]	4.7 ± 1.2 [c]	2.6 ± 1.0	23.4 ± 4.6
CD8/CD25	7.0 ± 2.4	2.3 ± 0.4	2.4 ± 0.8	23.0 ± 5.0
CD4/CD69	6.3 ± 1.4	4.5 ± 1.1	2.5 ± 0.5	23.1 ± 1.5
CD8/CD69	8.2 ± 1.7	7.6 ± 1.9 [d]	8.5 ± 3.0 [d]	32.1 ± 3.7 [d]

a: cells were harvested at 96 h (CD25) and 24 h (CD69) and assayed by flow cytometry; *b*: numbers represent the double staining-positive cells (mean ± SD) obtained in the stimulated cultures subtracted from the values of the non-stimulated cells; *c*: indicate Student's t-test significant differences when compared to CD8/CD25 ($p < 0.01$); *d*: CD4/CD69 ($p < 0.05$); *e*: Mtb antigens at 10 µg/ml, PHA at 1%.

tion and macrophage activation (Flynn & Chan 2001). Previous studies have shown overall agreement with CD25 or CD69 and proliferation assays when a recall specific antigen was used (Maino et al. 1995, Caruso et al. 1997). In addition, human PBMC sensitized to PPD responded to in vitro stimulation with increased CD69 expression and Th-1 type cytokine production (Skoberne et al. 2000). In the present study as related to Mtb antigen primed cells, enhanced detection of CD69 was observed around 24 h and of CD25 after 96 h. The data paralleled IFNγ production in these same cultures (not shown), indicating that both methods are in agreement and are sufficiently sensitive. The differential response induced by ferritin when compared to PPD and Ag85B were likewise of interest. In addition, CD69 was generally detected on CD8+ T cells. This preferential expression has already been described (Santamaria et al. 1992, Maino et al. 1995). In fact, this particular receptor may actually play an important role in activation signal transduction and stimulation of the cytotoxic machinery in T cells (Lanier & Phillips 1988). Thus, the Mtb Ags tested to date may also have the capacity to maturate the lytic granules of CD8+ T cells. Complementary experiments in TB patients are now in progress to confirm those data.

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