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Elevated percentage of perforin positive cells in active pulmonary tuberculosis

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***Background & objectives*: Perforin is one of the major effector molecules of cytotoxic cells associated with killing of cells harbouring intracellular bacterial infection. The precise role of perforin positive cells in tuberculosis still remains controversial. The present study was done to determine the number of circulating $CD4^+$ and $CD8^+$ perforin positive cells to assess the level of cytotoxic response against *Mycobacterium tuberculosis* in patients with pulmonary tuberculosis.**

***Methods*: Intracellular perforin and surface CD4 and CD8 staining of peripheral blood lymphocytes was done using specific monoclonal antibodies and enumerated using flowcytometry.**

***Results*: A significantly decreased total lymphocytes ($P<0.01$), CD4 ($P<0.001$) and CD8 ($P<0.01$) lymphocyte counts in PTB patients was observed compared to normal healthy individuals (NHS). Intracellular perforin staining showed significantly elevated percentages of total ($P<0.05$) and CD8 ($P<0.01$) perforin positive cells in PTB patients compared to NHS. However, the absolute counts of total, CD4 and CD8 cells positive for perforin were similar in patients and NHS.**

***Interpretation & conclusion*: Our results suggest that during active stage of pulmonary tuberculosis there was an increased percentage of CD8 cells positive for perforin, irrespective of their absolute counts. Further, $CD8^+$ perforin positive cells may have increased cytolytic activity against *M. tuberculosis* in active pulmonary tuberculosis.**

Key words CD4 - CD8 - perforin positive cells - pulmonary tuberculosis

Mycobacterium tuberculosis, the most successful intracellular pathogen accounts for more deaths worldwide than any other infectious pathogen[1]. Very few pathogens induce a broad spectrum of immune response as *M. tuberculosis*. Effective immunity to intracellular bacterial infection often requires the lysis of infected cells as well as killing of the pathogen. The role of cytotoxic cells is appreciated in other intracellular bacterial infections such as *Listeria monocytogenes* but its precise role in tuberculosis remains controversial[2,3]. It is postulated that lysis of infected macrophages could facilitate

the translocation of *M. tuberculosis* from incapacitated cells to more proficient effector cells[4].

Perforin is a 70 kDa pore forming glycoprotein stored and released by the lytic granules of cytotoxic killer cells [CD8 cells, natural killer (NK) cells, cytotoxic CD4 cells and γδ-T cells] on the target cell membrane. Infected target macrophages are lysed by the granule mediated perforin mechanism of apoptosis that co-deliver bactericidal proteins like granulysin[5]. It is shown that lysis of infected human dendritic cells and macrophages by CD8 cells specific for *M. tuberculosis* antigen reduced the intracellular bacterial numbers and the killing was perforin dependent[6,7].

The present study was aimed to delineate the level of CD4 and CD8 positive perforin positive cells to understand the cytolytic activity against *M. tuberculosis* in patients with pulmonary tuberculosis (PTB).

Material & Methods

The present study is part of a major ongoing project, cleared by the Institutional Ethical Committee. During the study period (January - May 2004), 30 pulmonary tuberculosis patients (mean age ± SE: 30.6 ± 1.2 yr) attending Tuberculosis Research Centre, Chennai, and 25 normal healthy volunteers who were willing to participate in the study were included. The patients with respiratory symptoms and radiographic abnormalities suggestive of pulmonary tuberculosis were confirmed by sputum positivity for *M. tuberculosis* by both smear and culture. All were HIV negative and studied before the commencement of anti-tuberculosis drug treatment. Informed consent was obtained from each patient before the collection of blood sample. Of the 30 patients included, 20 were males and 10 were females. The normal healthy group (17 males, 8 femals, mean age ± SE 25.9 ± 1.7 yr) included the volunteers who were clinically normal and free from viral diseases at the time of blood sample collection.

Peripheral blood was obtained by venipuncture. About 0.5 ml of blood was heparinized and used for haematological profile using electron profile counter (ABX micros CE Haematalgie, France), intracellular perforin, CD4 and CD8 staining.

Flowcytometric detection of intracellular perforin in whole blood was performed as described earlier[8]. The leucocytes of the heparinized blood (75 µl) were stained for surface CD4 or CD8 antigens using fluorescein isothiocyanate (FITC) conjugated antibody (Becton-Dickinson, USA). CD16 staining was also done in four normal healthy subjects. Red blood cells (RBC) were then lysed with RBC lysis solution (Becton-Dickinson, USA). The leucocytes were fixed in 4 per cent paraformaldehyde for 10 min and permeabilized at 4°C for 10 min with cold phosphate buffered saline (PBS) containing 1 per cent foetal calf serum (FCS) and 0.1 per cent saponin. Intracellular perforin staining was performed by incubating the cells with phycoerythrin (PE) or FITC labeled perforin antibody (Becton Dickinson, USA) at 4°C for 30 min. Stained cells were washed twice in cold PBS and then acquired in Becton Dickinson FACSort flowcytometer and analysed using 'Cell Quest Pro' Software. By scatter pattern, 10,000 gated events in the lymphogate were counted.

Student's 't' test was performed using 'Microsoft Excel 2000' software to find the significance of perforin positive cell numbers between the two study groups.

Results & Discussion

Significant decrease in total lymphocytes ($P<0.01$) and total $CD4^+$ ($P<0.001$) and $CD8^+$ cell ($P<0.01$) count was observed in PTB patients compared to normal healthy subjects (NHS). The absolute number of perforin positive total CD4, CD8 and CD4/CD8 double negative cells were similar in both PTB patients and NHS (Fig. A).

However, a significantly increased percentage of total perforin positive cells and CD8 perforin positive cells was observed in PTB patients as compared to NHS [Total: 24.5 ± 1.7 vs 20.7 ± 1.2%, ($P<0.05$); CD8: 12.7 ± 2.3 vs 9.4 ± 0.6%, ($P<0.01$)]. No major difference in the percentage of perforin positive CD4 and CD4/CD8 double negative cells was seen in the two groups (Fig. B).

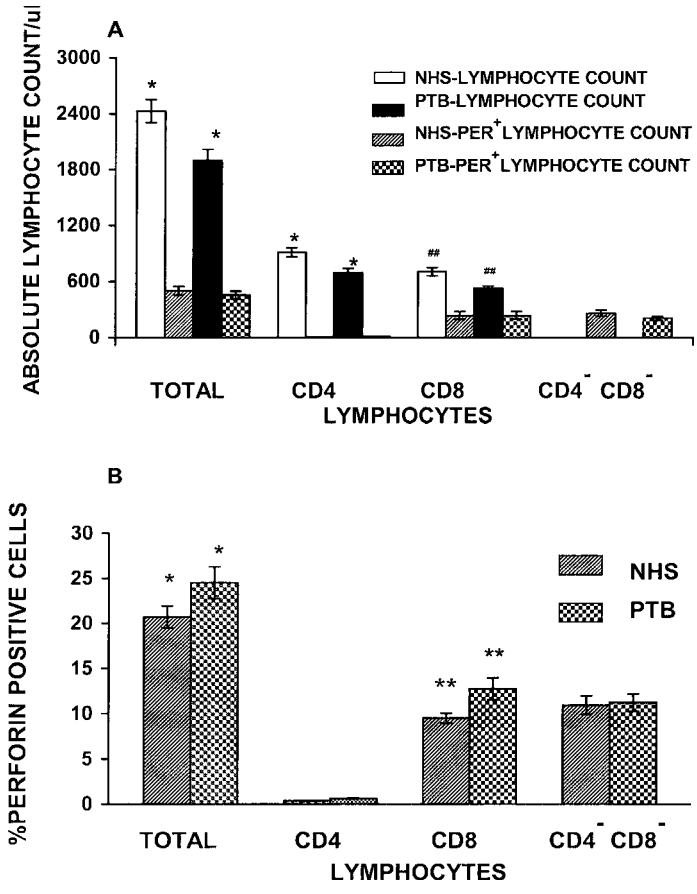

Fig.(A). Absolute counts of lymphocytes per microlitre in the peripheral blood of normals (NHS) and patients (PTB). Total lymphocytes (P<0.01*), perforin positive cells, $CD4^+$ cells (P=0.001**), $CD8^+$ cells (P<0.01#) CD8 perforin double positive cells, CD 16 perforin double positive cells. **B.** Immunophenotype of perforin positive cells among peripheral blood white cells in NHS and PTB. Significantly increased percentage of total perforin positive cells (P<0.05*), and $CD8^+$ $perforin^+$ cells (P=0.01**) was observed in PTB when compared to NHS. Results are expressed as mean ± SE.

$CD8^+$ cytolytic T-lymphocytes have been implicated in protective immune response against human tuberculosis. In the present study, significantly increased percentage of total perforin positive cells and CD8 perforin positive cells was observed in PTB patients compared to NHS. Although a significant increase in percentage of perforin positive CD8 cells was observed in patients, no difference in the absolute number of perforin positive lymphocytes in peripheral blood was seen due to decrease in the number of lymphocytes in PTB patients. This suggests that the cytolytic granules of $CD8^+$ and to certain extent $CD4^+$ cells are activated in pulmonary tuberculosis, by live *M. tuberculosis* and their antigenic components. Human *in vitro* studies have shown that $CD8^+$ T cells become activated and show cytolytic activity when stimulated with live *M. bovis* BCG and *M. tuberculosis* H37Rv[9,10]. A study carried out in Gambian population reported an increase in $CD8^+$ T cell activation and perforin production in response to *M. tuberculosis* H37Ra in normals when compared to tuberculosis patients[11].

CD4/CD8 double negative cells positive for perforin are predominantly natural killer (NK) cells[12]. However, CD4/CD8 double negative cells also include γδ T cells that also show natural killer activity similar to NK cells and express perforin. About 1-5 per cent of peripheral T cells are γδ cells. CD16 is a surface marker present on both NK cells and γδ T cells and is a component of Fcγ R-III which mediates antibody dependent cell cytotoxicity. An increase in NK cell (CD16/56) number has been reported earlier in pulmonary tuberculosis[13]. However, in the present study, we observed no

significant increase in CD4/CD8 double negative cells expressing perforin in PTB patients, a majority of which are NK cells. Earlier reports show that NK cells respond to *M. tuberculosis* infection but play a minimal role in protection[14].

In the present study, the observed increase in percentage of perforin positive cells of PTB patients may be due to perforin producing CD8 cells rather than NK cells or γδ T cells. Thus, in spite of decrease in the absolute number of $CD8^+$ cells, there was no decrease in total and $CD8^+$ cells expressing perforin in PTB patients. The present study suggests that higher percentage of circulating perforin positive CD8 cells in patients may serve as an evidence for the activation of acquired immune response and the cytolytic role played by $CD8^+$ lymphocytes against *M. tuberculosis in vivo*.

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