Invitro cytokine response in BCG vaccinated and non-vaccinated children

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ABSTRACT

This study was conducted to understand the in vitro immune responses in terms of cytokine generation in response to stimulation with different antigens in lymphocytes from BCG vaccinated and nonvaccinated children and to find whether there exists any difference in the cytokine pattern in vaccinated and unvaccinated normal children and relate this to their Mantoux status. Peripheral Blood Mononuclear cells were stimulated with different mycobacterial antigens to see the invitro IFN-γ and IL-10 levels in BCG vaccinated and non-vaccinated Mantoux positive and negative children by ELISA. The invitro results showed a significant increase in IFN-γ levels and IL-10 levels in the supernatants of antigen stimulated PBMC than the control. The level of response of the cytokines was influenced both by the antigen used, as well as by the group to which the child belonged. TH1 response in a Mantoux positive group which has received BCG vaccination appears to be much lower than the BCG non-vaccinated Mantoux positive group. Thus BCG appears to suppress the innate immune response. This suppression was more in TH1 cytokine response. Thus, TH2 response appears to be maximum in non vaccinated Mantoux negative group and it was suppressed in BCG negative Mantoux positive group. The BCG vaccinated Mantoux positive and negative groups appear to be different with respect to the cytokine profile, irrespective of the antigen used for stimulation. They are also not similar to the non-vaccinated Mx positive or negative children. The responses in the vaccinated Mantoux positive group are very different from vaccinated Mantoux negative group. This difference is consistent across antigens.

Keywords: BCG vaccination, Cytokines, immune response, Mantoux, PBMC.

INTRODUCTION

The only vaccine currently available against Tuberculosis (TB), is the attenuated Mycobacterium bovis strain Bacillus Calmette-Guérin (BCG). BCG vaccination for the prevention of tuberculosis has been used in humans since 1921. The demonstrated efficacy of Mycobacterium bovis BCG vaccine against pulmonary tuberculosis varies widely in different populations. The prevailing hypothesis attributes this variation to interactions between the vaccine and the environmental Mycobacteria, but the precise mechanism has so far not been clarified.[1] The fact remains, that the mechanisms of immunogenesis following BCG vaccination are poorly understood.

Immunity to Tuberculosis has been the subject of study for over five decades, however the protective mechanisms mounted by the host against tuberculosis or against vaccination are ill understood. Although BCG Vaccination has been given for over a century, the mechanism by which it supposedly induces protective immunity is not known.[2] We do not even know what the markers of immunity to tuberculosis are. Immunological changes in terms of cytokines patterns has not been studied in four naturally vaccinated exposed population. BCG being a mycobacterium should normally induce a reaction to tuberculin, which can be expected to be lifelong. But, in a large proportion of individuals the tuberculin positivity following BCG Vaccination wanes. Although waning has been
demonstrated, the only real thing we know is that a majority of vaccinated individuals do not show any reaction. We do not know what this implies for protection from tuberculosis. Thus a large grey area regarding the protective efficacy needs to be studied. Cytokines are Immunoregulatory proteins, which modulate the immune response to intracellular pathogens. A person infected with *M.tuberculosis* supposedly produces a protective immune response as can be identified by induration to the tuberculin skin test and remains clinically well. However, it is the tuberculin positive individual who is at greater risk of tuberculosis in later life.[6] Does BCG produce the same (immunologic) changes in the body as a primary infection, or are these different mechanisms? Does a primary infection also act this way? If yes, why then are there so many more cases of Tuberculosis among those who are Mantoux positive? From the data available on cell mediated immune response in tuberculosis it is evident that multiple T-cell subsets recognize multiple mycobacterial antigen targets and the secretion of cytokines vary with the spectrum of tuberculosis infection. However, no attempt has been made to determine the profile of cytokines in vaccinated and non-vaccinated healthy children. It is necessary to study the antigen recognition pattern and the cytokine profile of vaccinated and non-vaccinated, tuberculin test positive and tuberculin test negative children in order to understand the immune responses to BCG vaccination.

MATERIALS AND METHODS

A retrospective study was conducted on 105 healthy children in the age group of 5-8yrs. Mantoux was given to all the children after obtaining their parents consent. The children were grouped into four as BCG vaccinated Mantoux positive and negative BCG non-vaccinated Mantoux positive and negative based on the presence of BCG vaccination scar and Mantoux reaction. The study followed the ethical guidelines of the Tamil Nadu Dr.M.G.R. Medical University, Chennai.

Mononuclear cell preparation

Eight to 10 ml of blood was collected from each child. Peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll-Hypaque density gradient centrifugation at 1800 rpm for 30 minutes. The cells were then washed with Hank’s Balanced Salt solution (HBSS) and RPMI 1640 (Sigma Chemical Co, St. Louis, Mo) at 1500 rpm for 10 minutes. The PBMC’s were constituted in RPMI 1640 at the ratio of 1 x 10^6 cells/ml. The medium was supplemented with 10% autologous serum and 2 mM L-glutamine (Sigma Chemical Co). The viability of the cells was assessed by trypan blue exclusion method.

Stimulation of mononuclear cells

The PBMC at the density of 1C10^6 cells/ml were cultured in 48 well plates (Costar, Cambridge, Mass) with or without antigens. The cells cultured in the absence of *M. tuberculosis* antigen served as control. The antigens used for the stimulation were purified protein derivative (PPD, Span diagnostics), Phytoheam Agglutinin (PHA, Gene, Bangalore), culture filtrate (CF) and BCG vaccine (King Institute, Guindy at the concentration of 10 mg/ml. The culture filtrate antigen was prepared at Tuberculosis Research Center laboratory by growing the standard *M. tuberculosis H37Rv* strain in Sauton’s medium for six weeks. The culture filtrate was concentrated and its protein content was estimated using protein estimation kit (Bangalore, Genei) and diluted to required concentration. The PBMC were cultured in the presence of these antigens. The supernatants were collected after 48h, when the cytokine levels were found to be highest in initial standardization experiments. The cell-free supernatants were stored at -80°C for the cytokine assessment.

Cytokine assay

The supernatants of PBMC were thawed at the time of cytokine estimation by ELISA. Measurement of IFN-γ and IL-10 was done using the sandwich ELISA kit, according to manufacturer’s instruction (BD). The average of the duplicate readings was taken as the final concentration and the levels were expressed as pg/ml.

Statistical analysis

Data are presented as the mean (±SE) in both text and in figures. Comparisons between groups were done by Wilcoxon rank sum test. *P* < 0.05 was considered to be statistically significant.

RESULTS

Cytokine response to antigen stimulation

In order to investigate the quantitative differences in the T-cell response in terms of *in vitro* cytokine secretion (such as IFN-γ, IL10) among the four study groups,(Gr-I BCG+Mx+, GrII-BCG+Mx-, GrIII BCG-Mx+, GrIVBCG-Mx-) the cytokine levels were estimated at 48 hours after stimulating the mononuclear cells with mycobacterial antigens. The difference in the cytokine response upon antigen stimulation with respect to control levels within each group is to find the pattern of cytokine response and the comparison of differences in cytokine
levels between the groups are explained to find the impact of vaccination and Mantoux status on the in vitro cytokine response to mycobacterial antigens. In this study, the secretion of pro-inflammatory cytokine IFN-γ was significantly higher for PPD stimulation than for other mycobacterial stimulation as expected (p-value 0.002, 0.023, 0.004, 0.001, K.W. 33.499). The response to BCG stimulation was significantly raised in Mantoux positive group irrespective of vaccination. (P-value 0.001 and 0.008) and for the CF stimulation the significance raise was observed only in non-vaccinated Mantoux Positive group (p-value 0.001). (Table-1). IL10 is less in the vaccinated groups compared to the unvaccinated group irrespective of Mantoux status. (p-value 0.001) (Table-2) IL-10 response to PPD appears to depend both on the group and on the antigen used for stimulation. (p-value 0.002, 0.006) It is lower in the Mantoux positive children as compared to Mantoux negative children. PPD elicited response was more among the Mantoux positive individual irrespective of vaccination. (p-value 0.001) Inversely BCG elicited response was more among vaccinated children irrespective of Mantoux status. (p-value 0.017) CF elicited response was not dependent on either vaccination or Mantoux status. (p-value, 0.400) Thus, there was a pattern of response with respect to the antigen used for stimulation.

### Table: 1 Invitro IFN-γ and IL-10 levels in BCG vaccinated Mantoux positive and negative children

<table>
<thead>
<tr>
<th></th>
<th>BCG vaccinated Mantoux positive group</th>
<th>Vaccinated Mantoux Negative group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>IL-10</td>
</tr>
<tr>
<td>Control</td>
<td>12±2</td>
<td>12±2</td>
</tr>
<tr>
<td>PHA</td>
<td>15±4</td>
<td>24±2</td>
</tr>
<tr>
<td>PPD</td>
<td>56±56</td>
<td>43±5</td>
</tr>
<tr>
<td>CF</td>
<td>15±1</td>
<td>110±11</td>
</tr>
<tr>
<td>BCG</td>
<td>37±4</td>
<td>65±11</td>
</tr>
</tbody>
</table>

### Table: 2 Invitro IFN-γ and IL-10 levels in BCG non-vaccinated Mantoux positive and Mantoux negative children.

<table>
<thead>
<tr>
<th></th>
<th>Non vaccinated Mantoux positive group</th>
<th>Nonvaccinated Mantoux Negative group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>IL-10</td>
</tr>
<tr>
<td>Control</td>
<td>19±5</td>
<td>32±6</td>
</tr>
<tr>
<td>PHA</td>
<td>44±8</td>
<td>108±15</td>
</tr>
<tr>
<td>PPD</td>
<td>48±5</td>
<td>92±15</td>
</tr>
<tr>
<td>CF</td>
<td>45±8</td>
<td>136±18</td>
</tr>
<tr>
<td>BCG</td>
<td>22±4</td>
<td>123±67</td>
</tr>
</tbody>
</table>

### DISCUSSION

There have been many hypotheses to explain the inadequate protective effect of BCG against tuberculosis as the lack of an effective stimulation of T cell populations. However, the immune response following BCG vaccination and its relation to the Mantoux status of the child (or adult) under natural conditions has not been studied so far. This study attempts to understand the profile of the two cytokines studied, and to see if demonstrable differences exist between groups. IFN-γ response is one of the well-recognized correlates of protective immune response. There are previous reports on the higher IFN-γ response exhibited by the Mantoux positive individuals (than in Mantoux negative) and its correlation with the DTH response. This study observation does not support this as even the Mantoux negative children demonstrated a significant IFN-γ response to PPD stimulation. Non-vaccinated Mantoux positive group alone showed a CF-specific IFN-γ response for which the reason is unknown. The BCG-specific IFN-γ response by non-vaccinated Mantoux positive subjects indicates the prior exposure to environmental mycobacteria that shares cross-reactive antigens with M. bovis. The response of vaccinated Mantoux positive subjects can be attributed to the presence of memory T-cells generated due to the prior vaccination. But a study reported that the secretion of IL-2 and IFN-γ by the PBMC was significantly (P<0.05) high in the vaccinated Mantoux positive’s than the unvaccinated children. And they observed that in majority of the BCG vaccinated children, the stimulation of specific TH1 cells seem to be considerably high, in short-term in vitro cultures, The lack of IFN-γ response by BCG vaccinated Mantoux negative may be due to absence of memory cells and indicates that the BCG vaccination is ineffective in these children. Because of the non-exposure to environmental mycobacteria and non-vaccination status, the BCG on-vaccinated Mantoux negative group did not show any BCG specific response. Higher levels of IFN-γ were detected in PPD stimulation in vaccinated and Mantoux positive groups showed that BCG vaccination converts the Mantoux reaction in children. Why this occurs only in some children is a point that should be studied further.

IL-10 cytokine has been considered as an anti-inflammatory cytokine, acting in the inactivation of macrophages through inhibiting the production of IFN-γ by T lymphocytes. The IL-10 activity minimizes the tissue damage...
occurring in the disease site by inhibiting the production of proinflammatory cytokines. In this study, the IL-10 secretion was significantly higher in all the stimulated conditions implying that the response of this cytokine is mycobacterial antigen-specific and is independent of the vaccination status and exposure to environmental mycobacteria. Notably, the IL-10 levels were high for the CF stimulated conditions in all the groups. The absence of difference in IL-10 levels in vaccinated and non-vaccinated children indicates that prior BCG vaccination does not alter IL-10 production. This is in accordance with the previous report by Das et al. in pre and post vaccinated adults.[5]

However the TH1 response in a Mantoux positive group which has received BCG vaccination appears to be much lower than the BCG non-vaccinated Mantoux positive group. Thus BCG appears to suppress the innate immune response. This suppression was more in TH1 cytokine response. Thus, TH2 response appears to be maximum in non vaccinated Mantoux negative group and it was suppressed in BCG negative Mantoux positive group. While no definite distinguishing pattern could be made out, the findings strongly suggest that the four groups are different from each other in terms of their ability to secrete cytokines in unstimulated cells as well as in response to external stimulation.

Acknowledgement
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REFERENCES