Cell-mediated immune responses and cytotoxicity to mycobacterial antigens in patients with tuberculous pleurisy in Brazil

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Abstract

Evaluating human immune response to defined Mycobacterium tuberculosis antigens in patients with different clinical forms of tuberculosis may help in elucidating pathogenesis and in vaccine development. In the present report we evaluated the lymphocyte proliferation, cytokine production and natural killer cell cytotoxicity as parameters to screen four mycobacterial recombinant antigens. Pleural fluid mononuclear cells (PFMC) and peripheral blood mononuclear cells (PBMC) from 13 HIV-negative patients with tuberculous pleurisy, living in a tropical region of Brazil were used in these assays. Crude M. tuberculosis antigen and recombinant 70-, 65- and 38-kDa mycobacterial antigens, induced greater proliferation in PFMC than in PBMC. IFN-γ, TNF-α, IL-4 and IL-10 were evaluated in the PFMC supernatants stimulated by these antigens. Both crude and 70-kDa antigens induced higher levels of IFN-γ, TNF-α and IL-10. There was a significant positive correlation between IFN-γ and the proliferative response induced by crude M. tuberculosis antigen, and an inverse correlation was identified between IL-10 and cell proliferation. IL-4 was not detected in the supernatants of pleural fluid mononuclear cell cultures stimulated by either crude, or recombinant antigens. TNF-α was detected in variable amounts in supernatants of PFMC

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stimulated by all antigens tested. Natural killer cytotoxicity was induced by both crude and 70-kDa antigen. Our results demonstrate that cells present at the site of disease recognized three of the antigens screened, as shown by lymphocyte proliferation and production of regulatory and inflammatory cytokines, and the results obtained with PFMC were consistently higher than those obtained with homologous PBMC. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Most cases, and the large burden, of tuberculosis (Tb) mortality will occur in developing tropical countries until the year 2000 (Dolin et al., 1994). In Brazil, approximately 100000 new cases of Tb are reported annually (Gerhardt and Hijjar, 1993). One possible strategy to control tuberculosis could be the development of new subunit vaccines against this disease.

The human immune response against tuberculosis is mainly controlled by cellular-mediated immunity by activated T-cells (Kaufmann, 1995), leading to augmented mycobactericidal macrophage mechanisms through the production of IFN-γ (Flynn et al., 1993). PPD (purified protein derivative), a mixture of low-molecular weight proteins from *Mycobacterium tuberculosis*, has been extensively used as a tool to study the human immune response against tuberculosis (Ribera et al., 1990; Fine et al., 1994). PPD stimulates the production of cytokines implicated in Th1 differentiation, such as IFN-γ, as well as other T-cell and macrophage cytokines (Barnes et al., 1993; Zhang et al., 1994). Evaluating the human response against defined mycobacterial antigens is important in understanding tuberculosis pathogenesis and may help in the development of new vaccines. Although studies have evaluated the immune responses of individual mycobacterial antigens in human cells, few reports have compared human lymphocyte proliferative responses and cytokine production elicited by different mycobacterial antigens (Havlir et al., 1991; Barnes et al., 1992; Schoel et al., 1992; Silver et al., 1995).

Tuberculous pleurisy (Tbp) is considered a benign clinical condition, since patients clear the infection even in the absence of treatment (Stead et al., 1955). In this condition the pleural compartment contains an exudate rich in mononuclear cells, as well as immune (Barnes et al., 1990, 1993) and non-immune soluble factors (Ocaña et al., 1986; Costa et al., 1995). PPD-stimulated pleural fluid cells isolated from Tbp patients produce higher amounts of IFN-γ than homologous peripheral blood mononuclear cells (Ribera et al., 1990). This local immune response orchestrated by T cells and by a cytokine network seems to be involved in the favorable outcome of Tbp patients and may represent the protective immune response responsible for controlling the spreading of *M. tuberculosis* infection (Ferrer, 1997). Antigens capable of stimulating lymphocytes from Tbp exudate may be implicated in this protective response.
In the present study, four mycobacterial recombinant antigens (10, 38, 65 and 70 kDa), which have been shown to stimulate PBMC, were chosen for a comparative evaluation with PFMC in a comprehensive study including measurement of cell proliferation, cytokine production and cytotoxic activity.

The 70-kDa antigen is a cytoplasmic protein present in *M. tuberculosis*, *M. leprae* and *M. bovis* (Mackenzie et al., 1991). Similar to the 70 kDa antigen, the 65-kDa one is also a heat shock protein highly preserved in *M. leprae*, *M. tuberculosis* and *M. bovis* (Shinnick et al., 1988). A third mycobacterial protein evaluated was the 38-kDa antigen (Andersen and Hansen, 1989; Torriani, 1990; Andersen et al., 1990). It is a lipoprotein apparently anchored in the cell membrane by a lipid moiety (Young and Garbe, 1991; Espitia et al., 1992). The 10-kDa antigen of *M. tuberculosis* is present also in *M. bovis* BCG and it induces DTH in *M. bovis*-vaccinated guinea pigs (Baird et al., 1989; Minden et al., 1986).

Our goal was to screen mycobacterial antigens according to immune response parameters, in an attempt to distinguish immune dominant antigens. The chosen antigens have been shown to induce human immune responses, but have not been compared in their relative immunogenicity in the same patients. The tests used were aimed to evaluate the elements related to immune protection (IFN-γ and TNF-α), immunopathology (TNF-α) or innate resistance (NK cells). Cells obtained from HIV-negative patients with pleural tuberculosis living in the state of Bahia, a tropical region of Brazil, were chosen to screen these antigens.

2. Patients, material and methods

2.1. Patients

Thirteen HIV-negative patients with tuberculous pleurisy (Tbp), and without previous anti-tuberculous therapy were included in this study. Tbp was diagnosed by the histopathological finding of granulomas with caseous necrosis in the pleural biopsies, or by a positive culture of *Mycobacterium tuberculosis* in the pleural fragment. Venous blood was collected as a routine procedure for laboratory tests. Pleural exudate cells were obtained from drained pleural fluid obtained routinely for diagnosis and as part of treatment of patients with tuberculous pleurisy.

2.2. Mononuclear cells preparation

Pleural fluid mononuclear cells (PFMC) and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Hypaque gradient (Pharmacia Biotech, Sweden). Cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (HyClone Lab, Inc., Logan, UT), penicillin (10 IU/ml), and streptomycin (10 µg/ml) (Gibco).
2.3. Crude M. tuberculosis and recombinant antigens

M. tuberculosis antigens were extracted from M. tuberculosis H37Rv strain (ATCC 27294) grown in 7H9 medium with ADC enrichment (Difco Laboratories, Detroit, MI). Soluble extracts were prepared by disruption of bacteria using a sonicator (Sonifier B-12, Branson, CT). Bacterial debris were removed by high-speed centrifugation in phosphate-buffered saline and filtered through a 0.2-μm filter. Protein concentration was determined by a protein microdetermination kit (Sigma, St. Louis, MO). The 10-, 38-, 65- and 70-kDa recombinant mycobacterial antigens were kindly provided by Dr J.D.A. van Embden through the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease (TDR), Mycobacterial Antigen Bank. Possible LPS contamination was removed by Detoxi Gel Endotoxin Removing Gel (Pierce, Rockford, IL) using 0.1 M ammonium bicarbonate buffer. The levels of endotoxin found in the preparation of recombinant proteins were lower than 0.001%.

2.4. Evaluation of cellular proliferative response

Cell concentration was adjusted to 1 × 10⁶ per ml and cultivated in triplicate (200 μl of cells per well in a flat-bottomed 96-well plate) for 5 days in the presence of 10 μg/ml of crude M. tuberculosis antigen or recombinant antigens. Pokeweed mitogen (PWM, Gibco-BRL) was used at 1:100 final dilution in RPMI medium in some cell cultures. Tetanus toxoid (TT, Syeth-Ayersh Lab., PA) was added in a concentration of 10 μg/ml. Both PWM and TT were added for 5 days in order to compare the proliferative response of peripheral blood cells to pleural fluid cells. The proliferative response was determined by measurement of [³H]thymidine (1 μCi/well; Amersham, UK) incorporated during the last 6 h of culture. Cells were harvested in a cell harvester (Flow Laboratories, Norway), and the radiation was measured with a β counter (Wallac 1409, Finland). Stimulation index (SI) was calculated as the ratio of the mean of proliferative response in CPM (counts per minute) in the presence of stimuli by the CPM of unstimulated cell cultures.

2.5. Cytokine measurement

For cytokine production, 3 × 10⁶ cells were cultivated in 1 ml of RPMI per well in 24-well plates for 48 h. Cells were maintained in a 5% CO₂ incubator at 37°C in the presence of 10 μg/ml of crude M. tuberculosis antigens, or in the presence of 70-, 65-, 38- or 10-kDa mycobacterial recombinant proteins. Cytokine concentrations were measured in the culture supernatants by ELISA. IL-4 and IL-10 levels were measured with a commercial assay (Quantikine, R&D, MN). IFN-γ was measured with Human IFN-γ ELISA kit (KHC 4022, Biosource Intl., CA). TNF-α was quantified using two sets of antibodies (anti-TNF-α, Mab1, and a biotinylated anti-TNF-α Mab11) and standard TNF-α protein purchased from PharMingen (San Diego, CA).
2.6. Cytotoxicity assay

For cytotoxic assays, $3 \times 10^6$ pleural fluid cells and peripheral blood cells were cultivated in 1 ml of RPMI per well in 24-well plates for 5 days in presence of 10 $\mu$g/ml of crude *M. tuberculosis* antigen or in the presence of 70-, 65-, 38- and 10-kDa recombinant antigens. Natural killer cytotoxic activity induced by mycobacterial antigens in blood and pleural fluid cells was measured using K562 as target cells in a 4-h $^{51}$Cr release assay. Effector cells were used at different ratios from 10 to 100:1 E:T ratio against K562 cells labeled with Na$_2^{51}$CrO$_4$ (CNEN-Brazil). Cytotoxic activity is expressed as the percentage of specific $^{51}$Cr release relative to the maximum release of $^{51}$Cr from target cells incubated with 1% of Triton X-100 and spontaneous release of $^{51}$Cr from target cells incubated without effector cells. The percentage of cytotoxic activity (% lysis) was calculated as:

$$\text{% lysis} = \left(\frac{\text{test} \ ^{51}\text{Cr release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}\right) \times 100.$$ 

2.7. Statistical analysis

Differences of cell proliferation and cytokine production induced by different antigens in PBMC or PFMC were compared by analysis of variance (ANOVA). Student’s paired $t$-test was applied to compare individual responses to the same antigen (PBMC versus PFMC). Linear correlations were calculated between proliferative response and cytokines, or between different cytokines. Graphics and statistical analysis were made with GraphPad Prism software version 2.00 (GraphPad Software, Inc., CA).

3. Results

3.1. Proliferative responses

The higher frequency of mycobacterial-sensitized cells in the pleural compartment was demonstrated by comparing the proliferative response of PBMC to PFMC against mycobacterial antigens and unrelated antigens, such as pokeweed and tetanus toxoid.

Fig. 1A shows that cells from pleural exudate had significantly greater proliferation when stimulated by crude *M. tuberculosis* antigen than homologous blood cells (paired Student’s $t$-test, $P = 0.005$). Conversely, blood cells had significantly greater ability to proliferate when stimulated by pokeweed in vitro than pleural fluid cells (paired Student’s $t$-test, $P = 0.03$). Proliferative responses to tetanus toxoid were similar in PFMC and PBMC (Fig. 1A).

The ability of PBMC and PFMC from patients with tuberculous pleurisy to proliferate against crude *M. tuberculosis* antigen and against the four recombinant antigens is shown in Fig. 1B. Proliferative responses to recombinant antigens were markedly enhanced in pleural fluid cells compared to peripheral blood cells. The
Fig. 1. (A) Comparison between peripheral blood mononuclear cells (PBMC) and pleural fluid mononuclear cells (PFMC) proliferative responses to crude *M. tuberculosis* antigen (M.tb), pokeweed mitogen (PWM) or tetanus toxoid (TT). Responses of PFMC were higher than those of PBMC to M.tb antigen (*P* = 0.005). PBMC responses were higher than to PWM (*P* = 0.003). No statistical difference was observed between the two populations to tetanus toxoid (TT). Values are expressed as stimulation index (SI), each point represents a patient. (B) PBMC or PFMC proliferative responses to crude (M.tb) or recombinant mycobacterial antigens (70, 65, 38 and 10 kDa). Bars represent means ± the comparison of proliferative responses of PFMC and PBMC to other recombinant mycobacterial antigens (70, 65, 38 and 10 kDa) is shown. The values are represented by means ± S.D. in cpm. Significant differences between PBMC and PFMC responses were observed to crude *M. tuberculosis* (*P* < 0.05), 70- (*P* < 0.01) and 38-kDa (*P* < 0.01) antigens.
Fig. 2. Linear correlations between proliferative response expressed as stimulation index (SI) and levels of IFN-γ (A), IL-10 (B) or TNF-α (C) measured by ELISA in the supernatants of PFMC stimulated by crude *M. tuberculosis* antigen. Positive and significant correlation was reached between proliferation and IFN-γ (A), and a negative correlation between proliferation and IL-10 (B). The correlation between IL-10 and IFN-γ (D). Correlation coefficient ($r$) and the level of significance ($P$) are shown for each panel. NS, non-significant.
Fig. 3. (Continued)
10-kDa antigen did not induce proliferation either in blood or in pleural fluid cells, exhibiting levels similar to those of unstimulated cells.

All three recombinant proteins (70, 65 and 38 kDa) induced levels of proliferation similar to that induced by total crude \textit{M. tuberculosis} antigen. The 65-kDa antigen apparently induced less proliferation than 38- or 70-kDa antigens, but the difference was not significant.

There is a positive and significant correlation ($r = 0.7$, $P < 0.01$) between cell proliferation and amount of IFN-$\gamma$ secreted by PFMC in response to crude \textit{M. tuberculosis} (Fig. 2A). A negative and significant correlation ($r = -0.84$, $P = 0.03$) was found between the cell proliferation and IL-10 secreted in response to this same antigen (Fig. 2B). Correlations between TNF-$\alpha$ and cell proliferation (Fig. 2C) did not reach statistical significance.

3.2. Cytokine production

IFN-$\gamma$ production by PFMC exposed to different antigens is shown in Fig. 3A. PFMC from nine out of 13 Tbp patients included in this study produced IFN-$\gamma$ in response to crude \textit{M. tuberculosis} antigen (1315 ± 379 pg/ml) and 11 out of 13 to the 70-kDa antigen (1021 ± 334 pg/ml), six patients produced IFN-$\gamma$ in response to the 65-kDa antigens, but only two responded either to the 38- or the 10-kDa antigens. Unstimulated cells did not produce IFN-$\gamma$. No statistical difference was found between the response to crude \textit{M. tuberculosis} antigen or to the 70-kDa antigen. For both the 70-kDa and crude antigens the IFN-$\gamma$ secreted varied from patient to patient. IFN-$\gamma$ production in response to \textit{M. tuberculosis} antigen was significantly higher than those induced by the 65-, 38- or 10-kDa antigens (ANOVA, $P < 0.05$).

TNF-$\alpha$ production by PFMC exhibited a large variation among patients, and was similar in response to the recombinant proteins and the crude antigen (Fig. 3B). No statistical difference was found among the levels of TNF-$\alpha$ induced by all tested antigens (ANOVA, $P = 0.1$).

The amount of IL-10 detected in the supernatant of PFMC is shown in Fig. 3C. IL-10 was detected in the supernatant of cells stimulated by crude antigen (six out of 10 patients) and by the 70-kDa antigen (seven out of 10 patients). Four out of 10 patients responded to the 65-kDa antigen, and only one of 10 to either the 38- or 10-kDa antigens.

A negative though non-significant correlation ($r = -0.54$, $P = 0.1$) was detected between levels of IL-10 and IFN-$\gamma$ secreted by PFMC in response to crude \textit{M. tuberculosis} antigen (Fig. 2D). Overall results reveal that PFMC have a high secretion of IFN-$\gamma$ and TNF-$\alpha$, and lower production of IL-10, in response to tested antigens.

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Fig. 3. PFMC production of IFN-$\gamma$ (A), TNF-$\alpha$ (B) and IL-10 (C) in response to crude \textit{M. tuberculosis} antigen (M.tb) or recombinant antigen stimulation. Symbols represent individual patients and the means of each group is represented by the horizontal line. IFN-$\gamma$, TNF-$\alpha$ and IL-10 concentrations were significantly higher ($P < 0.05$) when PFMC were stimulated by crude \textit{M. tuberculosis} antigen or 70-kDa antigens than with other recombinant antigens.
3.3. Natural killer cell cytotoxicity

The comparison of cytotoxicity mediated by NK cells from pleural fluid cells to peripheral blood cells stimulated by crude *M. tuberculosis* antigen or unstimulated cells is shown in Fig. 4A. PFMC stimulated by crude mycobacterial antigen had greater specific cytotoxicity against K562 than PBMC or unstimulated cells. The mean specific cytotoxicity \((n = 3)\) at the 100:1 E/T ratio was 60, 33, 22 and 12% for antigen-stimulated PFMC, unstimulated PFMC, antigen-stimulated PBMC, and unstimulated PBMC, respectively. The comparison between NK cells activity
induced by crude or mycobacterial recombinant antigens is shown in Fig. 4B. Crude *M. tuberculosis* or 70-kDa antigens were higher inducers of NK activity against K562 than the 65-kDa antigen.

The mean specific cytotoxicity \( (n = 6) \) at the 100:1 E/T ratio in six different patients was 59, 40, 16, and 6\% in response to crude, 70-, 65-kDa or unstimulated pleural fluid cells, respectively. The 10- and 38-kDa antigens induced NK cell cytotoxicity similar to those observed in unstimulated cells (data not shown).

4. Discussion

In order to screen mycobacterial antigens according to immune response parameters, in an attempt to distinguish immune dominant antigens, we tested the proliferative responses of PBMC and PFMC from patients with tuberculous pleurisy to these antigens. Mycobacteria-specific mononuclear cells are enriched within the inflamed pleura, being poorly responsive to mycobacteria-unrelated antigens. Our data confirms previous reports in the literature about compartmentalization of anti-tuberculous response in the pleural space (Fujiwara and Tsuyuguchi, 1986; Barnes et al., 1989), and add new information on natural killer cell activity in this compartment. NK activity detected in cells from pleural fluid was higher than in PBMC-stimulated with crude *M. tuberculosis* antigen. The greater proliferation of PFMC and the augmented NK activity in response to crude mycobacteria antigens may be related to the production of IFN-\( \gamma \). IFN-\( \gamma \) is the major cytokine secreted by lymphocytes and NK cells in response to these antigens, as well as, through IL-12 produced by *M. tuberculosis*-infected macrophages (Flesh et al., 1995; Ladef et al., 1997). IFN-\( \gamma \) and IL-12 have been detected in the pleural fluid from patients with Tbp (Barnes et al., 1990; Zhang et al., 1994). The proliferative responses to 10-, 38- and 65-kDa proteins have been shown to be higher in PFMC than PBMC (Mehra et al., 1995). In the same report 10- and 65-kDa proteins induced higher levels of IFN-\( \gamma \) in PBMC from PPD-positive individuals than from diseased patients (Mehra et al., 1995). In another report these antigens were tested against PBMC from Portuguese individuals with pulmonary tuberculosis, and the results obtained were comparable to our results obtained with Tbp patients living in a tropical area (Silveira et al., 1997).

The role of NK cells in tuberculosis remains unclear, but it has been shown that blood cells from healthy PPD responders and pulmonary tuberculosis patients show augmented NK activity after incubation with *M. tuberculosis* sonicated antigen (Restrepo et al., 1990). We demonstrated that cytotoxic activity mediated by NK cells was induced by crude *M. tuberculosis* antigen and by the 70-kDa antigen. The capacity of 70-kDa antigen for inducing NK cells activity had not been previously reported. NK cells are capable of lysing a variety of tumor-, virus- and parasite-infected cells and are also able to lyse mycobacteria-infected cells (Blanchard et al., 1989). Additionally, NK cells are powerful pro-
ducers of IFN-γ when stimulated by IL-12 and participate in innate resistance to protozoan infections (Scharton-Kersten and Sher, 1997). It is possible that part of IFN-γ detected in PFMC cultures exposed to crude and 70-kDa antigen is produced by NK cells. Induction of mycobacteria-specific and nonspecific cytotoxicity, as well as IFN-γ and TNF-α production by the 65-kDa antigen, have been reported, probably through IFN-γ secreted by cells stimulated by this protein (Ab et al., 1990; Friedland et al., 1993). Our results demonstrated that the 65-kDa antigen induced significant lymphocyte proliferation and IFN-γ production, indicating its integrity, but did not induce NK cell activity. However, in the present study the hsp 65-kDa antigen induced much lower levels of IFN-γ and TNF-α than the hsp 70-kDa or crude antigen. IFN-γ and TNF-α are essential cytokines for IL-12 production by M. bovis-infected macrophages (Inge et al., 1995). The demonstration that crude M. tuberculosis or 70-kDa antigens are able to induce IFN-γ, and TNF-α is suggestive of their involvement in IL-12 production, which itself activates NK cells to exert their lytic and secretory activities.

IL-10 mRNA and IL-10 protein are elevated in tuberculous pleural fluid (Barnes et al., 1993). T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection probably by overriding the effect of IFN-γ (Murray et al., 1997) and by suppressing TNF-α and nitric oxide produced by mycobacteria-infected macrophages (Marshall et al., 1997). In our series, IL-10 was detected in supernatants of PFMC stimulated by crude, 70- or 65-kDa antigens. Its presence was inversely correlated to cell proliferation in response to crude antigen.

TNF-α was detected in most supernatants of cells stimulated by crude M. tuberculosis, 38-, 65- or 70-kDa antigens. This cytokine is mainly produced by mycobacteria-infected macrophages, and it has been incriminated in the immunopathology of tuberculosis (Filley and Rook, 1991). Supernatants of NK cells or TNF-α alone can inhibit M. avium growth within macrophages (Bermudez et al., 1995). It is interesting to note that in our report TNF-α production was similarly induced by the 70-, 65-, 38- or 10-kDa antigens, whereas the first two antigens were much more potent in inducing other parameters of the immune response. In conclusion, all three mycobacterial heat shock proteins evaluated are recognized by mononuclear cells present in pleural effusion from patients with tuberculosis, as indicated by their abilities to induce cell proliferation and cytokine production. The antigens lead to high IFN-γ and TNF-α production and low production of IL-10, which may implicate them in macrophage activation mediated by PFMC.

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