

Basic Research

Studies completed

Role of Mannose binding Lectin (MBL) gene variants on immune functions in pulmonary tuberculosis

Background:

Mannose binding lectin is a calcium dependent serum lectin (protein) secreted by the liver. MBL binds mannose and N-acetyl glucosamine terminated glycoproteins of the pathogens and augments macrophage phagocytosis and plays an important role in innate immune functions against pathogens. Variant MBL genotypes have been shown to be associated with altered MBL level. Our earlier studies revealed the association of functional mutant homozygotes of MBL with susceptibility to TB.

Aim:

To assess the regulatory role of variant MBL genotypes on MBL level and immune functions.

Methods:

The study was carried out in 58 normal healthy subjects (NHS) and 48 pulmonary TB (PTB) patients. MBL genotyping was carried out by DNA based polymerase chain reaction (PCR) and dot-blotting technique. Macrophage phagocytosis with live *M. tuberculosis*, spontaneous and *M. tuberculosis* culture filtrate antigen (CFA) induced lymphoproliferative response, were studied.

Results

The study revealed a significant increase of MBL level in pulmonary TB patients than normal healthy subjects ($p=0.008$). A similar trend in

MBL level was also observed irrespective of the MBL genotypes studied. However, the MBL genotype AA (MBL-52, 54 & 57 wild homozygotes), is associated with high level of MBL than AO (MBL-52, 54 & 57 heterozygotes) and OO genotype (MBL- 52, 54 & 57 functional mutant homozygotes). OO genotype is associated with very low level of MBL production in normal subjects and pulmonary TB patients.

(AA vs AO : NHS : $p = 2.46 \times 10^{-6}$, PTB : $p = 1.23 \times 10^{-5}$; AA vs OO : NHS : $p = 3.3 \times 10^{-9}$, PTB : $p = 3.11 \times 10^{-9}$; AO vs OO : NHS : $p = 0.00059$, PTB : $p = 0.0013$) (Fig. 3)

The percentage phagocytosis of live *M. tuberculosis* by peripheral blood monocyte derived macrophage was significantly decreased in pulmonary TB patients as compared to normal subjects ($p=0.005$). Normal subjects with AA genotype showed a significantly lower phagocytosis than normals with AO (heterozygote) genotype ($p=0.046$). In pulmonary TB patients, no difference in phagocytosis was observed among different genotypes (Fig. 4). The spontaneous and CFA induced lymphoproliferative responses were not influenced by the variant MBL genotypes.

Conclusion

The present study suggests that pulmonary TB patients have higher level of MBL with decreased macrophage phagocytosis with live *M. tuberculosis* and an opposite effect in normals. Pulmonary TB patients and normal subjects with functional mutant homozygotes (OO genotype) showed very low MBL level.

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Fig. 3. Serum MBL level in NHS and PTB patients with variant MBL genotypes

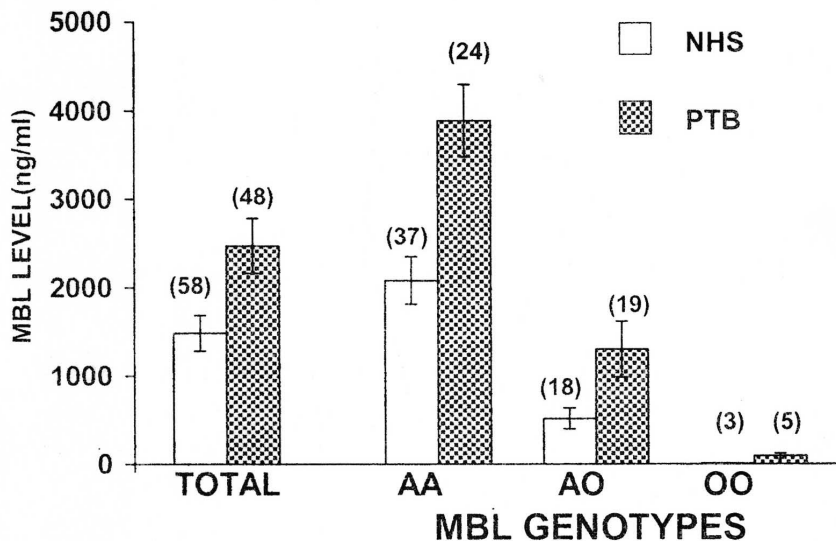
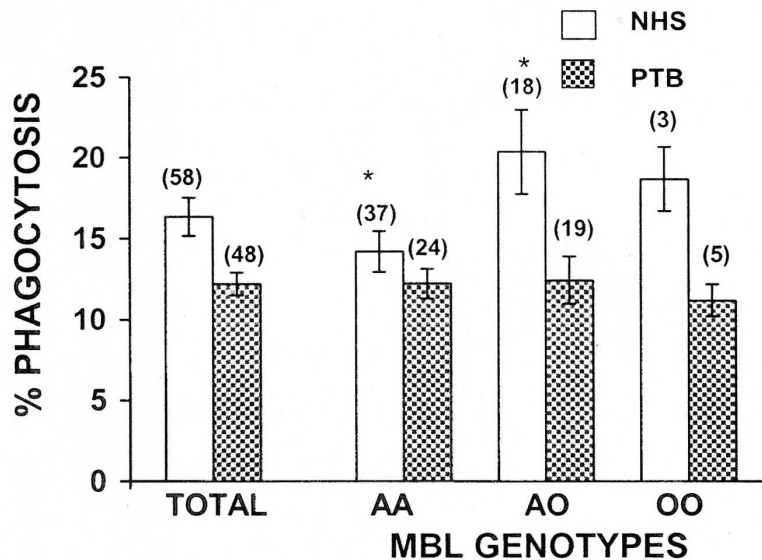


Fig. 4. Percentage phagocytosis in NHS PTB patients with variant MBL genotypes



Construction of recombinant BCG (rBCG) based HIV-1 epitope delivery system

Background:

AIDS is a global emergency. Reverse vaccinology is a fast emerging field which involves the identification of putative epitopes directly from the genomic sequences of the pathogens by *in silico* analysis. The lacunae in this novel approach is the lack of suitable “epitope-delivery vehicles” since the epitopes by themselves are poorly immunogenic. In this respect grafting an epitope from an immune unfriendly environment to an immune friendly environment enhances the immunogenicity of the epitopes and this forms the basic of ‘epitope grafting’.

Aim:

In the present project an ‘epitope-trap vector’ was constructed using *M.tuberculosis* chaperonin-10 (Cpn10) antigen as a carrier. Using this vector the HIV-1 PND epitope was expressed in rBCG. The immunogenicity of the rBCG vaccine was tested in a murine model.

Methods:

The three-dimensional structure of the Cpn-10 antigen was analyzed using INSIGHT II software and an extended loop region was identified for grafting the foreign epitope. Using this information two versions of the Cpn10-PND chimeric antigen were constructed and expressed in *M. smegmatis*:

1. The replacement chimera where the PND epitope replaces the Cpn10 loop and
2. The insertion chimera where the PND epitope is inserted into the Cpn10 loop

Based on the expression profile, p306CRC (Cpn10-PND replacement chimera in an episomal vector with homologous promoter) was electroporated into BCG Pasteur. Sub-cellular localization studies showed the presence of the

chimeric antigen in the cell wall, cytosol and culture filtrate but not in the cell membrane (Fig. 5). The immunogenicity of the recombinant BCG was evaluated in a murine model.

Results:

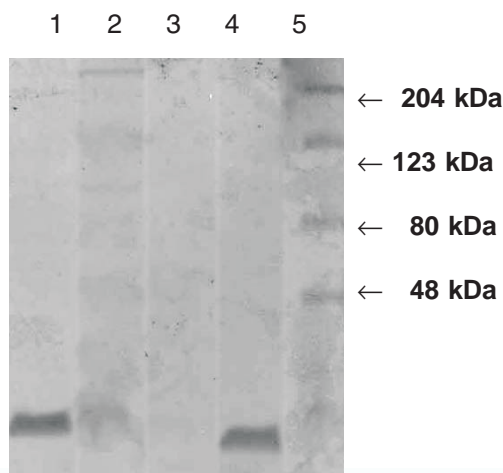
Vaccination with rBCG induced both cellular and humoral immune responses as measured by lymphocyte proliferation, delayed-type hypersensitivity (DTH) reaction and antibody production against the PND epitope (Fig 6). Isotype profiling of the serum anti-PND antibodies showed that all them were of IgG isotype with no IgA and IgM. Sub-isotype profiling showed the predominance of IgG2a and IgG2b sub-types indicating mixed response. The serum antibodies apart from recognizing the chimeric antigen also recognized other immunodominant antigens present in the culture supernatant of the rBCG.

Conclusion:

rBCG based epitope delivery systems was constructed and found to offer novel avenues in the field of reverse vaccinology.

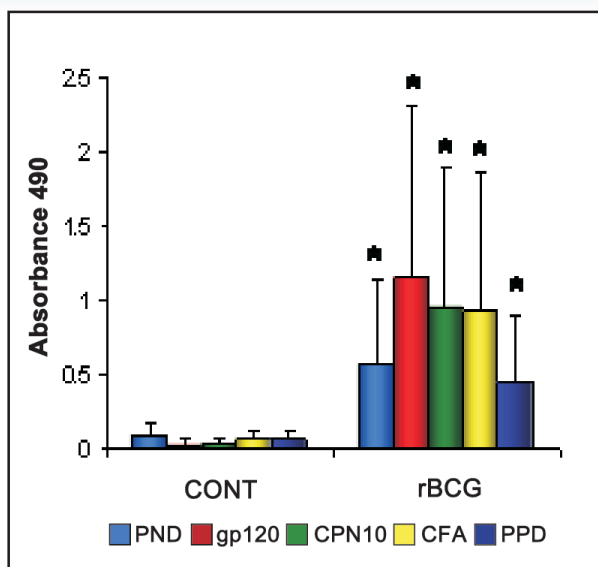
(Contact person: Mr.C.V. Aravindhan, e.mail: cvaravindhan@yahoo.co.uk)

Fig.5. Sub-cellular localization of Cpn10-PND chimeric antigen in rBCG



Lanes: 1-culture filtrate, 2-cell wall, 3-cell membrane, 4-cytosol and 5-Broad range marker.

Fig. 6. Humoral response induced by rBCG



Immune activation markers and their role in monitoring the course of HIV and tuberculosis

Background:

The association of HIV infection and TB is complex and bi-directional. Both HIV and TB via their actions on cell mediated immune responses, produce immune activation. We attempted to identify whether immune activation markers (neopterin, beta2-microglobulin, sTNFRI and sTNFRII) from serum samples obtained from clinically defined groups of patients could show relatively independent associations with TB or HIV disease in dually infected patients.

Methods:

The study population comprised of 42 HIV positive patients with active pulmonary tuberculosis (HIV+TB+), 37 HIV infected patients without tuberculosis (HIV+TB-), 38 HIV negative

patients with pulmonary tuberculosis (HIV-TB+) and 62 healthy volunteers (HIV-TB-).

Concentration of immune markers in plasma stored at -80°C were measured using commercially available capture ELISA kits, the immune markers were beta-2 microglobulin, neopterin, sTNF-R I and sTNF-R II.

Total and differential white blood cell counts were determined for all study subjects using the automated hematology analyzer. Percentage and absolute number of CD3, CD4 and CD8 T lymphocytes were measured by dual colour flow cytometry on a FACSsort Flow Cytometer using the Cell Quest software.

Results:

The mean CD4% of the HIV+TB+ group was $10.3 \pm 0.8\%$ while the HIV+TB- group was $16.7 \pm 1.3\%$. Table IX shows the level of the four activation markers, at baseline. Levels of activation markers TNF-RI, TNF-RII, beta-2 microglobulin and serum neopterin were elevated in all the patient groups except in the HIV+TB- group with less advanced disease (CD4 cell counts $> 200 \text{ cells/mm}^3$). There was a significant negative correlation between the CD4 count and the level of all the four immune activation markers. Table X shows the levels of immune activation markers before and at the completion of anti-TB treatment. Significant reduction of sTNF-RI and neopterin levels was seen in the HIV+TB+ group with advanced disease as well as in the TB group. The level of beta-2 microglobulin decreased significantly only in the HIV+TB+ group with advanced disease.

sTNF-R II levels did not show any significant changes after completion of anti-TB treatment in any of the patient groups studied.

Conclusion:

Although co-infection with TB and HIV produced a broad activation of the immune system, we have not been able to establish a definitive and independent marker for HIV and TB. None of the four markers studied was specific for disease progression of HIV and TB separately.

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Studies in Progress:

Immune response in tuberculous pleuritis: Differential T-helper cell response in tuberculous pleuritis by intracellular cytokine studies

Background:

Among the many clinical manifestations of TB, tuberculous pleuritis (TP) is of particular interest, as it offers protective immune response. In our earlier studies with pleural fluid mononuclear cells (PFMC), both Th1 and Th2 types of cytokines could be measured, in response

Table IX : Concentration of plasma immune activation markers in the study population at baseline

	HIV+TB+		HIV+TB-		HIV-TB+	HIV-TB-
	CD4 <200 (N=24)	CD4 >200 (N= 18)	CD4 <200 (N= 17)	CD4>200 (N= 20)	(N = 38)	(N =62)
sTNFR-1	4.2 ± 0.6 *	3.4 ± 0.5 *	1.6 ± 0.4 *	0.6 ± 0.1	3.6 ± 0.5 *	0.4 ± 0.1
sTNFR-2	0.6 ± 0.1 *	0.5 ± 0.1 *	0.6 ± 0.1 *	0.4 ± 0 *	0.5 ± 0 *	0.2 ± 0
Neopterin	24.4 ± 5.9 *	12.5 ± 2.7 *	14.9 ± 2.8 *	4.8 ± 2.1	13.8 ± 3.5 *	3.9 ± 0.6
Beta-2 microglubulin	5241.3± 1100.8 *	4642.8± 809.4 *	3664.1 ± 584.5	3393.6 ± 501.3	5979.2± 1892.9 *	1986.6±125.6

* p ≤ 0.05 against control (HIV-TB-group)

Table X : Concentration of plasma immune activation markers in TB patients with or without HIV infection before start and after completion of ATT

	HIV+TB+				HIV-TB+	
	CD4 < 200 (N =24)		CD4 > 200 (N= 18)		(N = 8)	
	0	6	0	6	0	6
sTNFR-1	4.2 ± 0.6 *	2.2 ± 0.3	3.4 ± 0.5	2.7 ± 0.4	3.5 ± 0.4 *	2.0 ± 0.3
sTNFR-2	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0	0.2 ± 0.0	0.4 ± 0.1
neopterin	24.4 ± 5.9 *	15.0 ± 5.9	12.5 ± 2.7	13.7 ± 4.4	not assayed	not assayed
beta-2 M	5241.3 ± 1100.8 *	3699.6 ± 291.9	4642.8 ± 809.4	4775.6 ± 76.5	6330.3 ± 1467.9	6461.8 ± 835.9

to *M. tuberculosis* antigens. In order to study the source of the Th1 and Th2 cytokines in pleural fluid, CD4+ T cells were purified. *Ex vivo* intracellular cytokine staining by flow cytometry was performed for interferon gamma (IFN- γ) and interleukin 4 (IL-4).

Aim:

To analyze the differential cytokine response in purified PFMC CD4+ T cells, by *ex vivo* and *in vitro* intracellular cytokine staining for IFN- γ and IL-4.

Methods:

The intracellular cytokine experiments were done in five TB pleuritis patients. The CD4+T cells were isolated from fresh peripheral blood mononuclear cells (PBMC) and PFMC and also from PFMC cultured with mycobacterial antigens [purified protein derivative (PPD), culture filtrate antigen (CFA), heat killed *M. tuberculosis* (MTB)]. The CD4+ T cells were positively selected using anti CD4 antibodies conjugated magnetic microbeads by MACS. Intracellular cytokine staining was done by fixing with paraformaldehyde and permeabilising the cells with 0.2% saponin. Fixed cells were stained with anti-human IFN- γ - FITC and anti-human IL-4 PE and analysed by FACS.

Results:

The *ex vivo* results on CD4+ T cells showed that there were cells producing IFN- γ alone, IL-4 alone or both. The percentage of IFN- γ producing cells were higher than the cells that secrete both IFN- γ and IL-4, and IL-4 alone (Fig. 7). Thus the pleural fluid T-helper cell response was predominantly of Th1 type. In contrast, *in vitro* stimulation of pleural fluid CD4+ T cells, with PPD, CF and heat killed MTB antigens, resulted in antigen specific increase in cells secreting IFN- γ alone and IL-4 alone. The double positive cells were also higher in

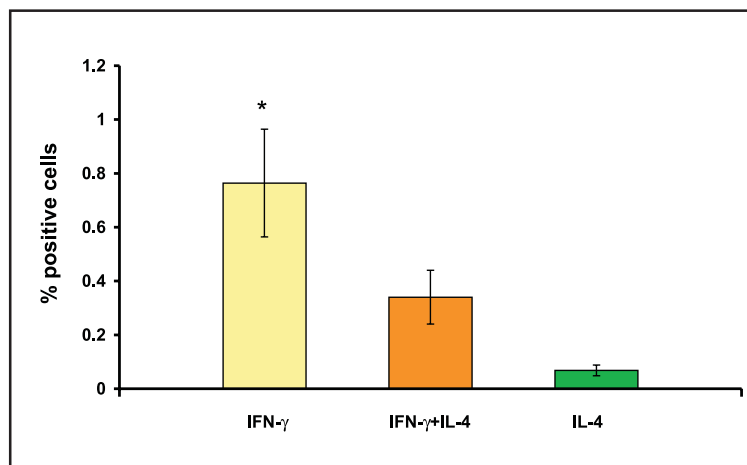
all cultured conditions, more so for PPD indicating that the response was predominantly of Th0 type (Fig. 8).

Conclusion:

There is a differential T-helper cell response in TP suggestive of Th1 *ex vivo* and Th0/mixed response *in vitro*. This confirms our previous observation of differential cytokine profiles in TP.

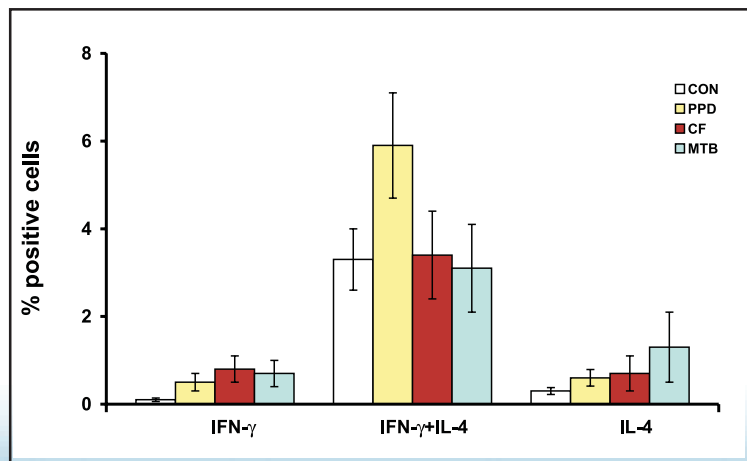
(Contact person : Ms.C. Prabha, e-mail: prabhapuffs@rediffmail.com & Dr. Sulochana D Das, e-mail: sulochanad@icmr.org.in)

Fig. 7. *Ex vivo* levels of intracellular cytokine in PFMC



* P<0.05 compared to other two groups

Fig. 8. *In vitro* levels of intracellular cytokine in PFMC



Molecular and immunological characterization of *M. tuberculosis* strains with single copy of IS6110: Th2 type of immune response observed in normal healthy individuals to S7 sonicate antigen

Background:

Different *M. tuberculosis* strains operate different immune evasion strategies for their survival in the host. This mainly depends on the virulence of the strain and the host immune responses. The most virulent strains are those actively involved in the transmission, are widely spread in the community and induce differential immune responses. The immune response was evaluated for sonicate antigen prepared from one such predominant strain (S7) of *M. tuberculosis* harboring single copy of IS6110 and actively involved in transmission.

Aim:

To evaluate the efficacy of the antigen S7 in modulating the immune response *in vitro* towards protection or otherwise in normal healthy PPD positive subjects.

Methods:

T-cell proliferative response to PPD, H37Rv and S7 antigens was studied. The cytokines IFN- γ , TNF- α , IL-12 and IL-4 in the culture supernatants and total IgG and IgA levels in the plasma of normal individuals were measured by ELISA.

Results:

Significant lymphoproliferative response and higher IFN- γ levels against PPD and H37Rv antigens were observed in PPD skin test positive normal individuals. The antigen S7 showed marginal T-cell proliferation, but did not induce IFN- γ secretion. Conversely, it induced significantly higher levels of IL-4 in normal

subjects. The macrophage cytokines IL-12 and TNF- α did not show S7 antigen specific stimulation (Figs. 9 & 10). The intracellular cytokine assay further confirmed the increase in IL-4+/CD4+ T-cells and decrease in IFN- γ /CD4+ T-cells after stimulation. The antibody response showed increase in IgG and IgA levels against this antigen in normal individuals.

Conclusion:

These observations suggest that antigen S7 modulates the *in vitro* immune response towards Th2 type by suppressing Th1 protective immune response in PPD skin test positive normal subjects. This leads to the speculation that some components of this sonicate antigen are associated with immunosuppressive response.

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Fig. 9. *In vitro* cytokine levels induced by MTB antigens in healthy PPD positive individuals

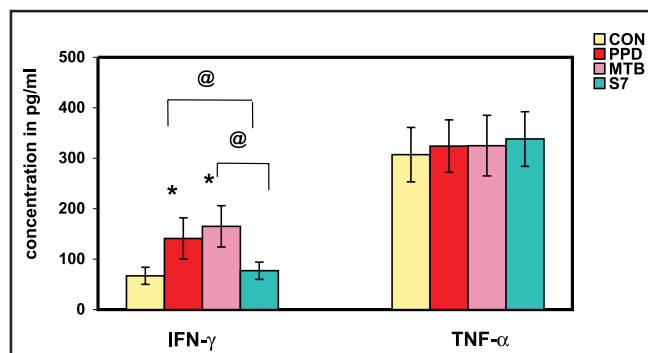
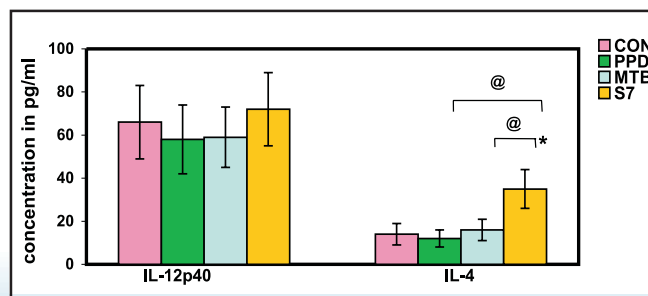


Fig. 10. *In vitro* cytokine levels induced by MTB antigens in healthy PPD positive individuals



*@ p < 0.05

Inflammatory response and apoptosis of polymorphonuclear neutrophils by prevalent strains of *M. tuberculosis*

Background:

Macrophages and polymorphonuclear neutrophils (PMN) are the professional phagocytes involved in antibacterial defense. The PMN influx, the first line of defense, occurs as the early response to curtail the mycobacterial infection. Chemokines stimulate the migration of PMN from circulation to the site of infection. *M. tuberculosis* induced activation leads to proinflammatory response and apoptosis of PMN.

Aim:

Two prevalent strains of *M. tuberculosis* (S7 and S10) showing differential immune response in PPD skin test positive subjects were selected for this study. The aim was to study the efficacy of these strains to induce apoptosis and modulate the expression of surface molecules and cytokine secretion in PMN of TB patients.

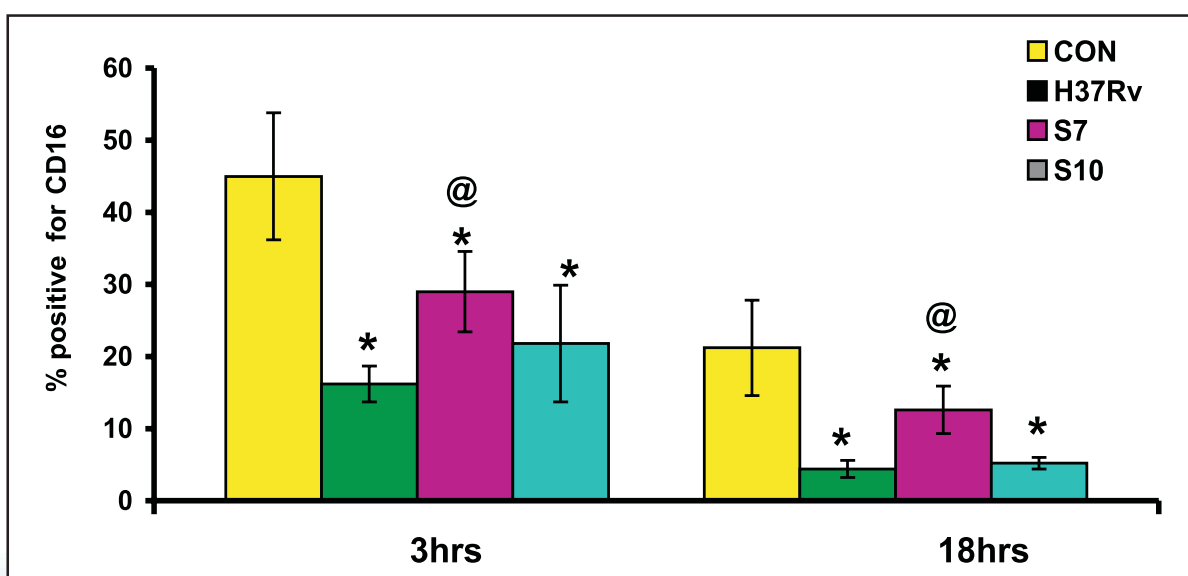
Methods:

PMN were isolated from red blood cell (RBC) pellet obtained from Ficoll-Hypaque gradient centrifugation and further subjected to sedimentation in 3% Dextran. PMN were infected with various mycobacterial strains (S7, S10, and H37Rv) at multiplicity of infection (MOI) of 3:1 and incubated for 3 and 18 hrs. The phagocytic index, percentage of apoptotic neutrophils (Annexin V positive), cell phenotypes (CD16 and CD69 by FACS) and cytokines (TNF- α and IL-1 β by ELISA) were assessed.

Results:

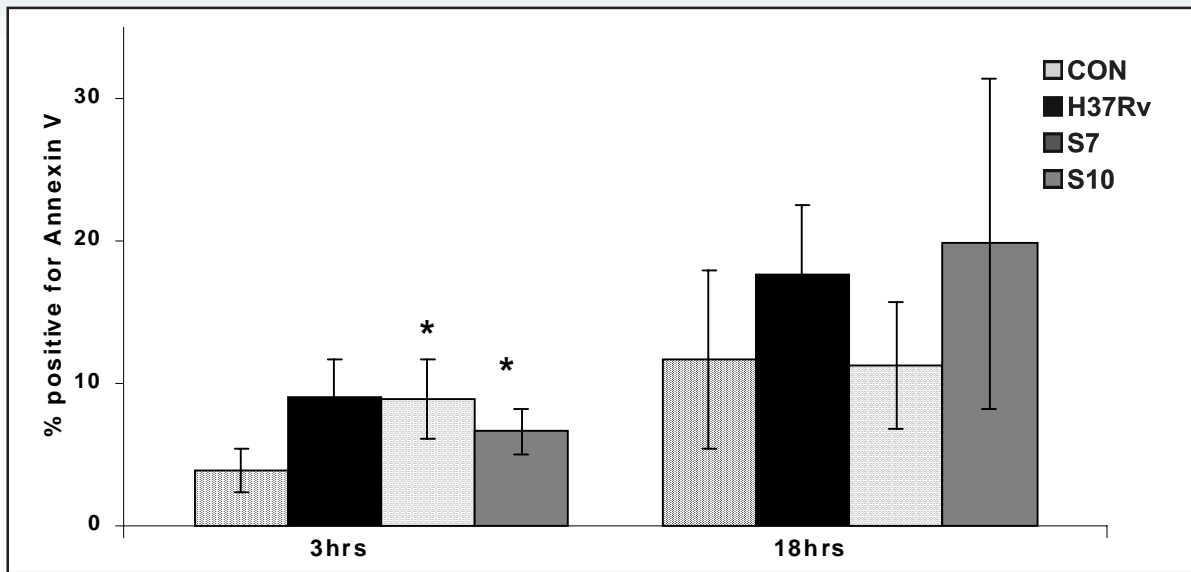
A significant increase in Annexin V positive cells with corresponding decrease in CD16 and CD69 expression was observed with S7 and S10 strains when compared to uninfected control after 3 hrs of infection. Further decrease in CD16 expression was observed at 18 hrs but no significant change in Annexin V positivity. When compared to H37Rv, S7 showed high CD16 expression at both the time points, but high CD69

Fig. 11. CD16 expression in TB-PMN after infection



* p < 0.05, compared to control
@ p < 0.05, compared to H37Rv

Fig. 12. Differential induction of apoptosis in TB-PMN



* p < 0.05, compared to control

@ p < 0.05, compared to H37Rv

expression only at 3hrs (Fig. 11 &12). There was no significant change in TNF- α production by the infected PMN at both the time points. Only S7, showed a significant increase in IL-1 β at 18 hrs when compared with control.

Conclusion:

All *M. tuberculosis* strains showed apoptosis of TB-PMN in a pro-inflammatory cytokines dependent manner. Clinical strains down-regulated CD16 expression and inhibited *de novo* synthesis of an early activation marker, CD69 on TB-PMN. These strains also showed a significant increase in apoptosis after infection thereby reducing the number of phagocytes and escaping from the intracellular lytic microenvironment. Thus clinical isolates were able to inhibit the early activation of neutrophils and thin out the killing mechanisms for their own survival.

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Identification of immunoreactive T-cell antigens of *M. tuberculosis* through proteomic techniques

Background:

Even though effective chemotherapy is available for treatment of TB, there are practical difficulties in ensuring the desired high cure rate, due to many factors. Immuno-prophylactic measures using vaccines is an alternative approach for control.

A limited number of attempts to screen human responses to separated antigens have demonstrated that there are still numerous uncharacterized antigens of various molecular masses to be evaluated. Moreover, a systematic approach to test the antigens purified by two-dimensional (2-D) preparative separations, in human subjects has not been attempted so far.

Aims:

The major aim of the proposed project is to identify a set of immunologically relevant T-cell

antigens and evaluate the response to these antigens in patients with TB and controls.

The main objectives are:

1. To carry out preparative 2-D electrophoresis and purify antigens of *M. tuberculosis*
2. To identify the immunologically relevant T cell antigens by comparing the *in vitro* proliferative response and cytokine (IFN- γ) response in tuberculous and control subjects.
3. To characterize the identified antigens using proteomics approaches.

Methods:

The study subjects are as follows:

1. Apparently healthy household contacts (HHC) from families where there is at least one case of sputum positive pulmonary TB living in the same household. TB will be ruled out in this group during the time of blood collection and hence considered “Protected”.
2. Newly diagnosed adult pulmonary tuberculosis cases in the age group of 16-50 yrs. They form the “susceptible” group.

The methods to be followed are as follows:

1. 2-D Preparatory separation of antigenic fractions

Fig. 13. Analysis of IEF fractions of CFA by 1D and 2D SDS-PAGE



2. Proliferative response and IFN- γ response will be studied using purified antigenic fractions.

Results:

The Culture Filtrate Antigen (CFA) has been subjected to First dimension Preparatory Isoelectric Focusing (IEF) in fluid phase and Second dimension Preparatory SDS-PAGE and Whole Gel Elution (WGE), for the preparation of antigenic fractions

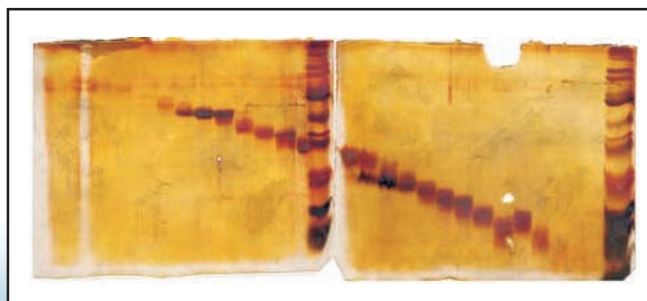
Figure 13 shows the Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) pattern of one of the 20 fractions separated by IEF. The number of bands (1-D) seen in each fraction ranged from 10 to 20. The number of spots (2-D) seen in each fraction ranged from 20 to 260. The pI of the 20 fractions ranged from 3.0 to 12.5.

Each of the 20 IEF fractions were again separated by WGE into 30 fractions. A representative picture (Fig. 14) of one of the fractions is shown.

Since a large number of fraction will have to be tested in each blood sample, we have standardized a whole blood (1:10 dilution) assay, for proliferation and IFN- γ secretion.

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Fig. 14. Analysis of Whole Gel eluted IEF fraction by 1-D SDS-PAGE



Role of HLA-DR2 on immune functions in pulmonary tuberculosis

Background:

Earlier studies revealed the association of HLA-DR2 with susceptibility to pulmonary TB. In continuation, the present study has been planned.

Aim:

To understand the role of HLA-DR2 on the immune mechanism of TB susceptibility.

Methods:

The study will be carried out in 60 pulmonary TB patients and 60 normal healthy volunteers. DNA typing of HLA-DR, enumeration of perforin positive cells by flow cytometry, macrophage phagocytosis, *M. tuberculosis* antigen induced lymphoproliferation and cytokine production will be studied.

The above immune functions were carried out in 40 normal subjects and 40 pulmonary TB patients.

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Human Leucocyte Antigen (HLA) and non-HLA gene polymorphism studies in HIV and HIV-TB patients

Background:

In developing nations, HIV-1 infection has increased the burden of TB, especially in populations where the prevalence of TB infection is high among young adults. The importance of host genetic factors (HLA and non-HLA) on susceptibility or resistance and the variability of disease progression to HIV-1 infection has been emphasized by many studies.

Aim:

To find out whether HLA genes, HLA haplotypes and non-HLA genes are associated with the susceptibility or resistance to HIV and HIV-TB.

Methods:

The study will be carried out in HIV negative TB negative (HIV-TB-) (n=150), HIV negative TB positive (HIV-TB+) (n=150), HIV positive TB negative (HIV+TB-) (n=150) and HIV positive TB positive (HIV+TB+) (n=150) groups. HLA-A and B antigen will be serologically determined. HLA-DR and -DQ and various non-HLA gene polymorphism will be studied by PCR based DNA typing.

During the period, HLA-A, B, -DR and -DQ gene polymorphisms were studied in 50 HIV-TB+ patients and 50 HIV+TB- / HIV+TB+ patients.

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Regulatory role of variant genotypes of vitamin D receptor and cytokine genes on cytokine response in pulmonary tuberculosis

Background

Our earlier studies revealed the regulatory role of vitamin D receptor (VDR) gene variants on vitamin D₃ modulated macrophage phagocytosis and lymphoproliferative responses.

Aim

To understand the regulatory role of VDR gene and cytokine gene variants on Th1 and Th2 type of cytokines in pulmonary TB.

Methods

The study will be carried out in 100 pulmonary TB patients and 100 normal healthy

volunteers. DNA typing of VDR gene and cytokine gene variants will be done using PCR-RFLP and dot-blotting techniques. Vitamin D₃ modulated antigen induced cytokines and granzyme positive cells will be studied.

During the year, 10 normal subjects and 10 pulmonary TB patients have been studied.

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Purification and characterization of a serine/threonine protein kinase-PknE of *M. tuberculosis* H37Rv

Background:

Serine/threonine protein kinases form important components of signal transduction elements along with the two-component systems in mycobacteria. They are “eukaryotic like”, control important physiological processes in the cell and are involved in various aspects involving stress responses, development and pathogenicity. The biochemical characterization of these kinases would aid in better understanding of the ions required for autophosphorylation, kinase inhibition, substrate phosphorylation and cross reactivity with eukaryotic antibodies.

Aim:

To clone, express, purify and characterize the PknE protein of *M. tuberculosis* H37Rv.

Methods:

E.coli DH5 α , GJ1158 and BL21 (DE3) were the strains that were used in the study. The *pknE* gene was PCR amplified and cloned into pRSETb (full length gene) and into pGEX-5X3 (kinase domain). The recombinant proteins were purified

using Nickel ion affinity chromatography and Glutathione affinity chromatography respectively. *In vitro* phosphorylation assays were carried out using γ -P32 labelled ATP and various divalent cations (Fig.15). Phosphoamino acid analysis and substrate phosphorylation were assessed using anti-phosphoserine and anti-phosphothreonine antibodies. Kinase inhibitors were used to generate the inhibition profile for the recombinant proteins. Antibodies against eukaryotic kinases were used to determine cross-reactivity with the purified protein (Fig. 16).

Results:

Recombinant proteins were expressed and purified either with a 6X histidine tag (full length enzyme) or a Glutathione-S-Transferase tag (kinase domain alone) and were found to correspond to a molecular weight of ~67kDa and ~68kDa respectively. PknE was found to be a magnesium or a manganese requiring enzyme. The purified proteins were found to autophosphorylate at serine and threonine residues and also phosphorylated the exogenous substrate, rabbit muscle enolase. They were capable of being inhibited by staurosporine and H7. The recombinant PknE protein cross-reacted with SAPK/JNK and phospho-SAPK/JNK antibody. Thus one of the eleven serine threonine protein kinases, PknE was cloned, expressed, purified and characterized biochemically. The purified protein showed a phosphorylation profile and kinase inhibition profile similar to the kinases of other bacteria.

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Fig. 15. *In vitro* autophosphorylation assays for the recombinant PknE proteins

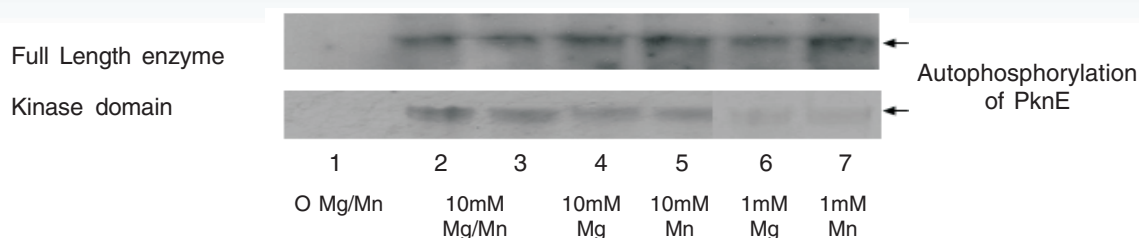
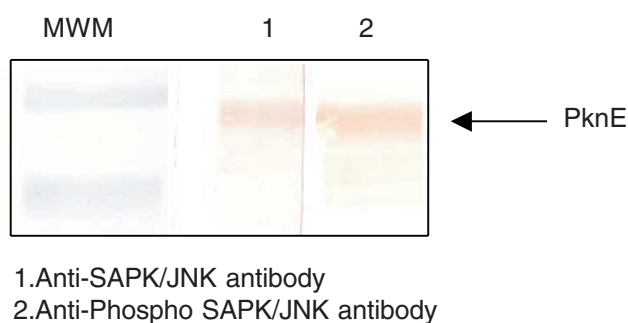


Fig. 16. Cross reactivity with eukaryotic protein kinase antibodies



Characterization of *cis* and *trans* acting factors regulating *M. smegmatis* acetamidase operon

Background:

Acetamidase was the first highly inducible gene found in *M. smegmatis*. The gene is part of an operon which has four open reading frames apart from the acetamidase gene and it has a complex regulation. By PCR mediated deletion mutagenesis we have identified several *cis* acting elements involved in the regulation of this operon. Specific binding of proteins to these upstream regulatory regions was observed in mobility shift assays. Further cold chase mobility shift experiments have been carried out to further confirm the specificity of binding.

Aim:

To identify the *trans*-acting factors that bind to these upstream *cis*-acting elements which controls acetamidase regulation.

Methods:

Whole cell lysate was obtained by bead beating. Ammonium sulfate fractionation was carried out using standard protocol Mobility Shift assay was carried out using the various fractions and the radiolabelled probes.

Results:

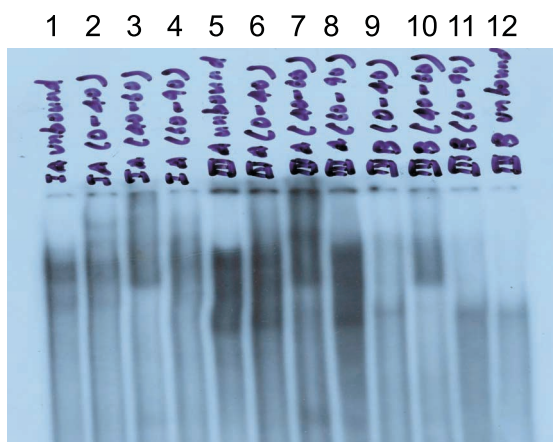
The fraction obtained between 40% and 60% was found to contain DNA binding activity based on mobility shift assays (Fig. 17). Figure 18 shows

the whole cell lysate fractionated by ammonium sulfate precipitation. An affinity chromatographic approach is being used to purify and characterize these DNA binding factors. As an alternate approach cloning of the four upstream open reading frames is also being carried out to find out

whether these proteins are involved in regulation of acetamidase induction.

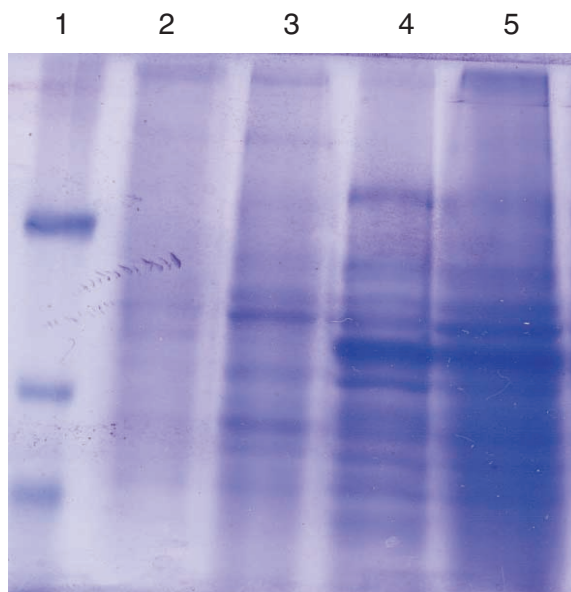
(Contact person : Mr.S. Dhinakaran, e-mail: dinakar_80@yahoo.co.in & Dr. Sujatha Narayanan, e-mail: sujathan@icmr.org.in)

Fig. 17. Mobility shift assay of ammonium sulfate fractions



Lanes 7 and 10 corresponding to ammonium sulfate fractions 40-60% showing shift in mobility.

Fig. 18. SDS-PAGE of fractions



Lane 1: Low Range marker
 Lane 2: Fraction 0-40%
 Lane 3: Fraction 40-60%
 Lane 4: Fraction 60-90%
 Lane 5: Whole Cell Lysate

Characterization of possible targets of Serine/ Threonine protein kinases of *M.tuberculosis*

Background:

Serine/ Threonine Protein Kinases (STPKs) are a novel class of mycobacterial proteins found to be involved in the signal transduction machinery in addition to the two component systems (TCS). These STPKs are being explored for the development of new drug targets and they have also been implicated in virulence and persistence of the pathogen. Two STPKs-PknI and PknE are being characterized in the laboratory.

Aim:

To identify the targets of the STPK-PknI.

Methods:

E.coli DH5 α and BL21 (DE3) were the

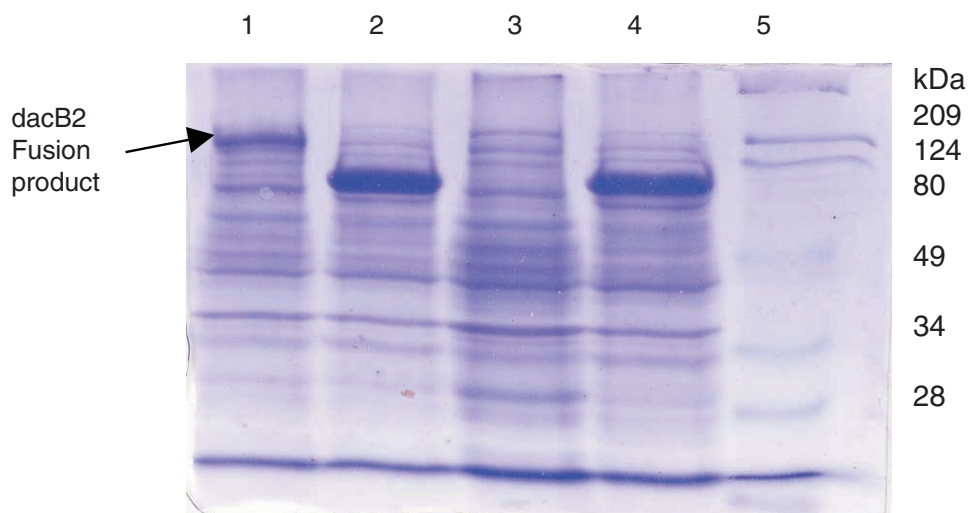
strains that were used in the study. Primers were designed using the genome sequence of *M. tuberculosis* H₃₇Rv for the three genes *dacB2*, *ftsY* and *ffh* (probable targets of *pknI*).

Results:

PCR amplification was done for *dacB2*, *ftsY* and *ffh*. The three PCR products were cloned in a TA vector. Sub-cloning of *dacB2* in an expression vector pET43.1a was done (Fig. 19). DNA sequencing of the cloned insert was carried out. Expression studies were performed following standard protocols. Purification of the recombinant protein is in progress.

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Fig. 19. SDS PAGE profile showing expression of *dacB2*



The role of complement activation and antibody in the early interaction between *M. tuberculosis* and macrophages

Background:

The complement system, which represents a chief component of innate immunity not only participates in inflammation but also act to enhance adaptive immune response. The initial interaction between host macrophage and *M. tuberculosis* is an important first step in the pathogenesis of tuberculosis and is mediated by specific macrophage receptors and ligands present on the surface of *M. tuberculosis*. The survival and replication of *M. tuberculosis* within the host macrophage are documented features of the pathogenesis of tuberculosis. However the means by which *M. tuberculosis* evades being killed by macrophages remain unclear. The phagocytosis, immune regulation, cytokine production, and effectors mechanism may all contribute innate immune responses. In the present study investigate that the antibody could modulate complement activation and determine the interaction of *M. tuberculosis* with the macrophage.

Aim:

To study whether antibodies could modulate complement activation and determine the interaction of *M. tuberculosis* with the macrophages.

Methods:

1. *M. tuberculosis* treated with complement (classical or alternative pathway) in the presence and absence of IgM or IgG antibodies against *M. tuberculosis*.
2. Investigate the receptor mediated route of entry of *M. tuberculosis* with in the macrophages.
3. Human macrophage will be used phagocytosis of tubercle bacilli and quantitative assessment of free radicals and different cytokine levels.
4. Intracellular viability of the tubercle bacilli will be assessed as cfu obtained from lysed macrophages.
5. The macrophage thus treated will be exposed to calcitriol and induction of apoptosis will be assed.

Results:

Methods to culture macrophages from peripheral blood monocytes, phagocytosis of *M. tuberculosis* by macrophages and estimation of viable counts of tubercle bacilli after phagocytosis by the macrophages have been standardized. Antibody purification and standardization of methods to estimate cytokines and free radicals are in progress.

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Serum and tissue complement profile in *M. tuberculosis* infection

Background:

The complement system as part of the innate immune system influences the immune response to *Mycobacterium tuberculosis* by affecting both the cellular and humoral immune responses. This system is involved not only in the early interaction between the host and the pathogen but also plays an important role in the inflammatory reactions associated with the disease and acts as an acute phase reactant. Therefore, this exercise is designed to delineate the exact role of the complement system in tuberculosis.

Aim:

To study the complement profile in the patients with active and healed pulmonary tuberculosis and to observe the status of the complement system during treatment by sequential estimation of the complement proteins and their activated fragments.

Methods:

Participants

The study subjects will comprise of 25 patients with active, smear positive pulmonary tuberculosis, 25 subjects who have completed the full anti-tuberculosis treatment regimen and 25 normal healthy volunteers as controls.

1. The levels of complement proteins (C3, C4, C2, Factor B, MBL) and their activation fragments (C3d, iC3b, C4d, Bb, SC5b-9) will be measured by ELISA.
2. The functional efficiency of the activated complement cascade will be assessed by haemolytic complement through Classical Pathway (CH50) and the Alternative Pathway (AH50).
3. The altered expression of membrane bound complement effector proteins (CR1, CR2, CR3, CR4) in PBMC will be studied by Flow Cytometry.
4. The role of complement components in the clearance of circulating immune complexes will be measured by ELISA.
5. The expression of mRNA of complement proteins will be studied by PCR, RT-PCR
6. To study the relationship between the innate immune response and the individual

susceptibility to tuberculosis, genotyping of the class III antigens (C2, C4, Factor B) will be performed by RFLP-PCR.

Results:

The above mentioned techniques are being standardized and samples are being collected.

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Complement activation by strains of mycobacteria: Wild type and gene disrupted *M. tuberculosis*

Background:

Various pathogens and microbes including *M. tuberculosis* and *M. bovis* BCG are known to activate the complement system, which plays an essential role during the early interactions between *M. tuberculosis* and the human host. However, not much is known about the activating potential of various gene-disrupted *M. tuberculosis*. Hence, the present study is designed to investigate the interaction of the complement system with these genetically modified mycobacteria.

Aim:

To study the various effects triggered on the complement system by these strains and to study the changes induced in the protein component of the organisms by the complement system.

Following are the strains that are to be used: MtpA and MtpB (Tyrosine phosphatases A and B), a strain lacking both tyrosine phosphatases, VirS knockout, devR knockout and their respective complemented strains.

Methods:

1. Complement activation: Solid phase ELISA would be performed to assess the activating potential of complement by these strains.
2. Two dimensional electrophoresis and western blotting: Will be performed to assess the changes in the protein components of the bacteria after interacting with the complement system.

Results:

ELISA methods to assess complement activation by mycobacteria at the level of C3 and C4 have been carried out with MptpB and Erdman strains. Experiments with devR have been started.

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