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Original Article *Ind. J. Tub., 1999, 46, 21*

ANTIBODY AND LYMPHOCYTE RESPONSES TO *MYCOBACTERIUM* TUBERCULOSIS CULTURE FILTRATE ANTIGENS IN ACTIVE AND. QUIESCENT (CURED) PULMONARY TUBERCULOSIS

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(Received on 29.6.98; Accepted on 28.8.98)

Summary : Humoral and lymphocyte responses to *Mycobacterium tuberculosis* culture filtrate antigens were studied in active pulmonary tuberculosis (ATB) cases (n = 62), inactive (cured/quiescent) tuberculosis (ITB) patients (n = 62) and healthy control subjects (n = 60).

Active tuberculosis patients showed very high antibody titre to *M. tuberculosis* culture filtrate antigens as compared to ITB and control subjects. *M. tuberculosis* antigens from 17 to 80 kDa were recognised by the plasma of all ATB and ITB patients as well as control subjects. However, the 38, 32-34, 30-31 and 27 kDa antigens were recognised more by the ATB patients as compared to the control subjects while the 64/66 kDa antigen was mostly recognised by the cured patients. Increased lymphocyte responses were seen with increasing concentrations of M. *tuberculosis* culture filtrate antigens and mitogens such as Phytohaemagglutinin (PHA) and Concanavalin-A (Con-A) in ATB and ITB patients as well as healthy control subjects. However, a low or suppressed lymphocyte response to PHA, Con-A and *M. tuberculosis* culture filtrate antigens was seen in ATB patients compared to ITB patients and control subjects.

The study suggests that during the active stage of the disease, the humoral immune response is augmented but the antigen and mitogen induced lymphocyte response (an in vitro correlate of CMI response) is suppressed. This further suggests that the humoral immune response regulates the CMI response during the active stage of the disease; when the disease is cured, the antibody response declines and the lymphocyte response to antigens and mitogens increases to the same level as found in controls. This suggests that normal immune status gets restored in cured patients.

Key words *: Antibody response; Antigen recognition; Lymphocyte response; M. tuberculosis antigens; Mitogens; Pulmonary tuberculosis.*

INTRODUCTION

Tuberculosis is a chronic infectious disease regulated almost entirely by the cell-mediated immune response (CMI) of the host against *M. tuberculosis.* When bacillary antigens are present at low levels, the CMI response causes macrophages to accumulate, become activated and then destroy the bacilli. However, when bacillary antigens are present at high levels, the CMI response causes necrosis of tissues[1]. The immune response against *M. tuberculosis* involves both macrophages and lymphocytes. Dead organisms are broken down relatively rapidly within the granuloma, releasing large quantities of glycolipid and polysaccharide antigens into the draining lymphnodes, leading to a predominantly humoral immune response[2]. On the other hand, viable mycobactria release small quantities of glycoprotein antigens which induce a delayed type hypersensitivity (DTH) and cell-mediated immunity response[3]. As more and more tubercle bacilli are killed by the immune host, considerable quantities of glycolipid and polysaccharide antigens are released from the cell-wall of *M. tuberculosis.* These antigens are responsible for increasing humoral response (IgM, IgG, IgA)

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which usually peaks after the T-cell mediated immune response has declined[4].

A number of studies have been carried out to understand the immune response against *M. tuberculosis* during the active stage of the disease[5-12]. However, no attempt appears to have been made to understand the immune status (cell-mediated and humoral immunity) of patients after successful treatment (cured patients). The present study was aimed at the humoral immunity and lymphocyte response to *M. tuberculosis* culture filtrate antigens in active (ATB) as well as cured (ITB) patients and healthy control subjects. Moreover, lymphocyte response to mitogens such as Phytohaemagglutinin (PHA) and Concanavalin-A (Con-A) was also studied.

MATERIAL AND METHODS

Study subjects

Active tuberculosis (ATB) patients

Patients attending the Tuberculosis Research Centre (TRC), Chennai, with respiratory symptoms and radiographic abnormalities suggestive of pulmonary tuberculosis were studied. These patients had sputum positive for *M. tuberculosis* by smear and culture. Blood specimens were taken from them before the start of chemotherapy. Among the 62 ATB patients studied, there were 55 males (mean age ± S.E. = 38 ± 1.6) and 7 females (mean age ± S.E. = 29 ± 3.1).

Inactive (Cured) tuberculosis (ITB) patients

These patients had received short course anti-tuberculosis chemotherapy at TRC for 6-8 months' duration 10-15 years back. At the time of blood specimen collection, all these cured patients were rechecked to be quiescent. Of the 62 such patients studied, 44 were males and 18 were females. Mean age with SE was 42 ± 1.6 for males and 35 ± 1.9 for females.

Control subjects

Healthy spouses and other family contacts (n= 21) of cured tuberculosis patients and clinicians, social workers, health visitors, laboratory volunteers and other staff (n = 39) working at TRC for more than 3 years were studied. Of the 60 control subjects, 25 were males and 35 were females. Mean age with SE was 38 ± 1.7 for males and 37 ± 1.4 for females. The patients and their spouses were not consanguineous to each other, nor were the control subjects related to any of the patients. But, all belonged to the same ethnic origin and were living in and around Chennai.

Peripheral blood

Peripheral blood mononuclear cells (PBMC) were separated out from heparinised (20 units/ml) blood specimens using Ficoll-hypaque density gradient centrifugation[13]. Plasma was used for the estimation of IgG antibody titre and antigen recognition pattern against *M. tuberculosis* culture filtrate antigens. PBMC were used for studying lymphocyte response to *M. tuberculosis* antigens and mitogens.

M. tuberculosis culture filtrate (Antigen)

M. tuberculosis culture filtrate antigen was prepared as described earlier[14]. Briefly, $H_{37}Rv$ strain was grown for 6 weeks (late logarithmic phase) as a surface pellicle on Sauton's medium at 37°C. The culture was centrifuged and the supernatant fluid was filtered. The culture filtrate proteins were precipitated with 80% ammonium sulphate and dialysed against PBS (pH 7.4). The dialysed protein was lyophilised and resuspended in minimal volume of sterile PBS Its protein concentration was estimated and it was stored at -20°C until required. The antigen concentrations used for eliciting lymphocyte response were 0.1, 1.0 and 10.0 μg/ml of culture.

Mitogens

The mitogens Phytohaemagglutinin (PHA) (Wellcome Diagnostics, Dartford, U.K) and Concanavalin-A (Con-A) (Sigma Chemicals Co., St. Louis, U.S.A.) were used at 0.1, 1.0 and 10.0 μg/ml concentrations.

Enzyme linked immunosorbent assay (ELISA)

The IgG antibody titre against *M. tuberculosis* culture filtrate antigens was measured by ELISA as described earlier[14]. Antigen coated (5 μg/ml) ELISA plates (polyvinyl chloride) (Costar, Cambridge, Ma. USA) were incubated with serially diluted plasma specimens (from 1 : 5 to 1 : 20480) at 37°C. Rabbit anti-human IgG peroxidase conjugate (Bangalore Genet Pvt. Ltd., Bangalore) was used as the second antibody (1 : 1000) and incubated. Colour was developed using orthophenylenediamine (OPD 0.5 mg/ml) and 0.1% hydrogen peroxide as substrate. The optical density (OD) was measured at 490 nm in an ELISA reader (Titertek multiscan plus. MKII, Flow Laboratories International, Lugano, Switzerland). Twice the mean OD value of the blank was used as the cut-off point. Plasma specimens showing OD values above the cut-off point were taken as positive and the corresponding titre values were recorded.

Immunoblotting

Culture filtered antigen (50 μg/cm of gel) was loaded onto a 12.5% sodium dodecyl sulphate polyacrylamide slab gel electrophoresis (Protean Bio-Rad Laboratories. California, USA) using a discontinuous tris-buffered system as described elsewhere[15]. A molecular weight marker was run along with the antigen and stained with 0.5%. Coomasie brilliant blue (R250 - Bio-Rad Laboratories, California, USA). After electrophoresis, proteins were transferred electrophoretically to nitrocellulose paper (NCP) (Advanced Microdevices Pvt. Ltd., Ambala. India) by the method of Towbin *et al.,* [16] in a Blot Apparatus (Transblot Apparatus. Bio-Rad Laboratories, California, USA) for 1 hr at 65 mA constant current. The NCP strips were treated with the plasma specimens of patients (1 : 50 dilution) and rabbit anti-human IgG peroxidase conjugate as second antibody (1 : 1000 dilution), with hydrogen peroxide as substrate and colour was developed using 0.04% diaminobenzidine (Bio-Rad Laboratories, California, USA) in PBS as the chromogenic substance. One strip was included as positive control (hyper immune rabbit serum against *M. tuberculosis* $H_{37}Rv$ strain) in each set of experiments using anti-rabbit IgG peroxidase as second anti-body (1 : 5000).

Lymphocyte transformation test

The lymphocyte response of ATB, ITB and-control subjects to *M. tuberculosis* culture filtrate antigen was studied, as described earlier[14]. Briefly, 0.1 x 10^6 of PBMC in 200 μl was cultured with various concentrations (0.1, 1.0 and 10.0 μg/ml) of *M. tuberculosis* culture filtrate antigens, 10% autologous plasma for controls and ITB patients and serum for ATB patients. Autologous serum was used instead of plasma to avoid clotting. PBMC cells were cultured for 6 days in a 96, well 'U' bottom tissue culture plate (Coaster, Cambridge. Ma, USA), at 37°C and 5% carbon-dioxide in a CO, incubator (Flow Laboratories Ltd., Rickmansworth, Hertz, UK). Tritiated thymidine (^3H) (BARC, Trombay, Mumbai) was added to each well (1 μCl/well) 16 hrs before termination. The cells were harvested and the lymphocyte response to *M. tuberculosis* antigen was measured by tritium labelled thymidine uptake by the cells in a scintillation system using Beta counter (LKB, USA) and expressed as counts per minute (cpm). The mean cpm value of the triplicateculture was calculated and the stimulation index (SI) was derived using the formula :

$$SI = \frac{\text{Mean cpm of antigen stimulated cell cultures}}{\text{Mean cpm of cell cultures without antigen}}$$

Statistical Analysis

The results of antibody titres and lymphocyte responses are expressed as arithmetic mean ± S.E. Student's 't' test was used to assess the significance of the difference between means. Proportionality test was used for immunoblot analysis to asses the significance.

RESULTS

The IgG antibody titre against *M. tuberculosis* $H_{37}Rv$ culture filtrate antigens was

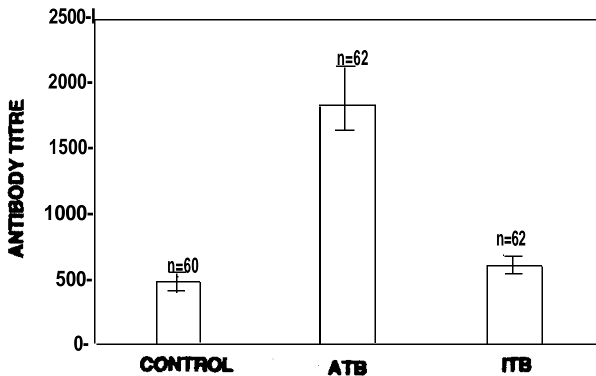

Fig. 1. Antibody titre to *M. tuberculosis* culture filtrate antigen in ATB, ITB patients and control subjects. Results are expressed as arithmetic mean ± S.E. The number of subjects studied was : Controls : n = 60; ATB : n=62; ITB : n=62
Controls Vs ATB P < 0.01;
Controls Vs ITB P < 0.01;
ATB Vs ITB P < 0.01

higher in ATB patients as compared to ITB patients and controls (Figure 1). Further, the antibody titre of ITB patients was higher compared to that of control subjects. The differences in the antibody titres were quite significant between the different groups studied (Controls Vs ATB - P < 0.01 : Controls Vs ITB - P < 0.01; ATB Vs ITB - P < 0.01).

Most of the *M. tuberculosis* culture filtered antigens, from 17 kDa to 80 kDa were recognised by the IgG antibodies present in the plasma of active and inactive TB patients. More inactive tuberculosis patients recognised 64/66 kDa antigen compared to the active TB patients (P < 0.01) and control subjects (P < 0.01). Both active and in active TB patients recognised 38 kDa antigens significantly more often compared to control subjects (ATB Vs Controls, P < 0.05 : ATB Vs ITB P < 0.05). Further, 30/31 and 32/34 kDa antigens were also recognised more often by ATB and ITB

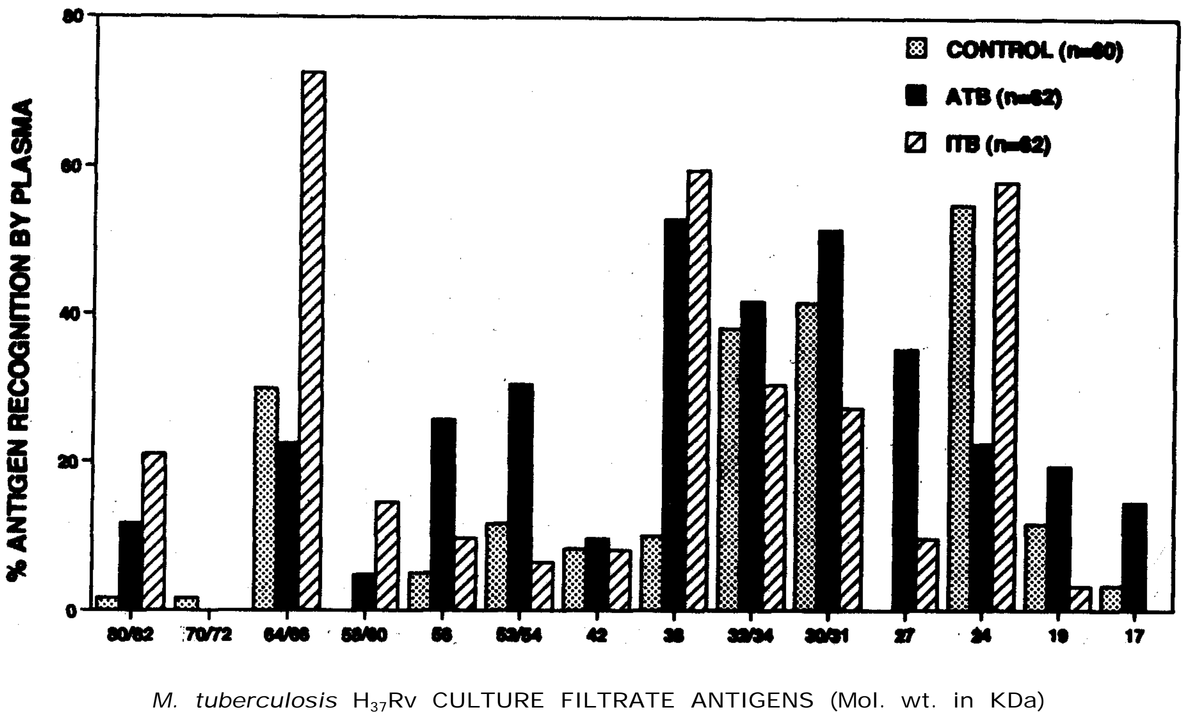

Fig. 2. *M. tuberculosis* culture filtrate antigen recognition pattern by the plasma of ATB and ITB patients and control subjects Percentage of subjects recognising the various antigens of *M. tuberculosis* culture filtrate is represented. The number of subjects studied in each group was : Controls : n = 60; ATB : n=62; ITB : n=62.

(i) Significant recognition of 52/54 kDa, 38 kDa, 27 kDa and 17 kDa antigens was seen:
Controls Vs ATB - P < 0.05; ITB Vs ATB-P < 0.05

(ii) Significant recognition of 64/66 kDa antigen was seen : Controls Vs ITB-P < 0.01; ATB Vs ITB-P < 0.01

patients compared with controls; 27 kDa antigen was recognised more often by active TB patients compared to ITB patients (P c 0.05) and control subjects ($P < 0.05$). The recognition of antigens 52-54 kDa, 38 kDa, 27 kDa and 17 kDa was significantly higher in ATB patients compared with ITB and control, subjects (Figure. 2).

An increased lymphocyte response with increasing concentrations (0.1 µg, 1.0 µg, and 10.0 µg) of *M. tuberculosis* culture filtrate antigen was seen in ATB, ITB patients and control subjects (Table). The lymphoproliferative responses of ITB patients to antigenic stimulation was significantly higher than controls and ATB patients at all the three (0.1 µg/ml, 1.0 µg/ml and 10.0 µg/ml) concentrations studied (Control Vs ATB-$P < 0.01$; Control Vs ITB-$P < 0.01$; ATB Vs ITB-$P < 0.01$).

The mean stimulation indices for PHA were significantly higher in controls compared with ATB patients at 0.1 µg/ml ($P < 0.01$) and 1.0 µg/ml concentrations ($P < 0.01$). There was not much difference at the higher concentration (10.0 µg/ml). Moreover, significantly higher lymphocyte response to PHA was seen in the ITB patients compared with ATB patients irrespective of the concentrations of PHA studied. Not much difference was seen among controls and patients at lower concentration (0.1 µg/ml) of Con-A but values were significantly higher in controls compared with ATB patients at higher concentrations (1.0 µg/ml and 10.0 µg $P < 0.01$). Further, ITB patients showed a significantly increased response to Con-A at 1.0 µg and 10.0 µg ($P < 0.01$) compared with ATB patients. The responses of ITB patients to Con-A concentration were similar to that of controls (Table).

A decreased lymphocyte response to *M. tuberculosis* antigens and mitogens was seen in all the concentrations studied in ATB patients compared to control subjects and ITB patients. However, Con-A at 0.1 µg concentration did

Table Lymphocyte response of healthy control subjects and pulmonary tuberculosis (ATB and ITB) patients to different concentrations of M. tuberculosis antigen and mitogens

Antigen/ Mitogen	Conc. (µg/ml)	Mean Stimulation Index ± SE		
		Control	ATB	ITB
	0.1	3.2 ± 0.4	1.4 ± 0.1	8.3 ± 1.7
M. tuberculosis antigen	1.0	5.9 ± 0.8	2.5 ± 0.4	17.3 ± 2.7
	10.0	11.5 ± 1.6	4.5 ± 0.9	31.7 ± 4.9
		(n = 58)	(n = 50)	(n = 44)
	0.1	10.8 ± 2.4	6.4 ± 2.7	13.2 ± 3.5
	1.0	82.1 ± 9.5	34.4 ± 7.4	98.8 ± 13.1
PHA	10.0	82.6 ± 9.4	61.6 ± 11.7	79.8 ± 12.1
		(n = 49)	(n = 21)	(n = 42)
	0.1	3.4 ± 0.8	3.7 ± 2.4	3.1 ± 0.4
	1.0	19.6 ± 3.8	5.0 ± 1.1	24.9 ± 5.0
Con-A	10.0	56.7 ± 6.6	21.1 ± 3.0	67.9 ± 9.4
		(n = 52)	(n = 27)	(n = 44)

1. Numbers in parentheses represent actual number of subjects studied.
2. MTb antigen (i) Control Vs ATB : $P < 0.01$; (10 µg/ml)
   (ii) Control Vs ITB : $P < 0.01$
   (iii) ATB Vs ITB : $P < 0.01$
   (P values are significant at 0.1 and 1.0 µg/ml. (P values not given)
3. PHA (i) Controls Vs ATB : $P < 0.01$ (1 µg/ml)
   (ii) ATB Vs ITB $P < 0.01$ (10 µg/ml)
4. Con-A (i) Controls Vs ATB : $P < 0.01$ (1 µg and 10 µg/ml)
   (ii) ATB Vs ITB : $P < 0.01$ (1 µg and 10 µg/ml)

not show any suppression. This decreased response was significant ($P < 0.01$) at 1.0 μg and 10.0 μg concentrations of either the antigen or the mitogens studied (Table).

DISCUSSION

In the present study, high antibody (IgG antibody) title to *M. tuberculosis* culture filtrate antigens was seen in ATB patients compared to control subjects and the cured (ITB) patients. Earlier studies had shown similar results[8,17]. This may be due to heavy bacillary (antigenic) load triggering high B cell response. It is well established that high antibody titre is elicited only during the secondary immune response as IgG antibodies. The antibody titre in the cured (ITB) patients was also significantly higher compared with control subjects, which shows persistence of high IgG antibody level even in the cured.

The antigen recognition pattern by these antibodies revealed that both patients and control subjects recognised the antigens, from 17 kDa to 80 kDa, as found in other studies also[18,19]. This suggests that most of the culture filtrate antigens are immunogenic.

Antibodies to 64-66 kDa antigens were seen in control subjects as well as patients. However, a larger proportion of ITB patients recognised this antigen compared with control subjects and ATB patients. Emmrich *et al.*[5] have demonstrated that epitopes of 65 kDa antigen are shared by bacteria such as *E. coli* and atypical mycobacteria. Thus, the 65 kDa antigen elicits a strong B cell response inducing antibodies controls in as well as in ATB and ITB patient groups.

The 38 kDa antigen is strongly recognised by active and inactive tuberculosis patients compared with the control subjects. Most smear positive pulmonary tuberculosis patients had a high antibody titre but recognition of 38 kDa antigen by inactive tuberculosis patients suggests that antibody levels are maintained for a long time. This suggests that the 38 kDa fraction of *M. tuberculosis* is a strong immunogen capable of evoking a strong B cell memory response.

Recognition of 32 - 34 and 30 - 31 kDa antigen fractions was found to be only slightly increased in patients compared with control subjects, unlike the 38 kDa antigen, and fewer treated patients recognised this antigen. Humoral immune response to antigen 85A and 85B (30 and 31 kDa) has been found to be much higher in active-tuberculosis patients compared with the controls[20] but not so in treated patients as in patients undergoing treatment[21].

We found in this study that 27 kDa fraction in *M. tuberculosis* culture filtrate antigen was recognised by active tuberculosis patients but not by control subjects. However, a small percentage of ITB patients also recognised this antigen. Since reports on the nature and immunogenecity of this antigen are scanty, more investigations are needed, as it may help in differentiating ATB patients from healthy subjects (immunodiagnosis).

Immunity in tuberculosis being a purely cell-mediated defence[7], lymphoproliferative response or mitogen stimulation have been widely used as *in vitro* correlates of cell-mediated immunity". In the present study, patients with active tuberculosis showed a low lymphocyte response to *M. tuberculosis* culture filtrate antigens and mitogens (PHA and Con-A) compared with control subjects and treated tuberculosis patients. A low T-cell responsiveness was observed in ATB patients even in respect of mitogen stimulation (Concanavalin-A). Low T lymphocyte reactivity is well documented in tuberculosis[11]. The low responsiveness may be attributed to the suppressive factors secreted by monocytes and lymphocyte[6]. Another possibility may be a shift from Thl (responsible for cell-mediated immunity) to Th2 (responsible for humoral immunity) type of cytokine response[9,12,23]. Further, preferential sequestration of antigen specific T cells into the infected areas[24] leads to their absence in peripheral blood. Such compartmentalisation has been described in patients with severe pulmonary and or miliary tuberculosis. Cured patients showed a higher response to *M. tuberculosis* culture filtrate antigens compared with control subjects, may be due to the presence of sensitised T-cell to *M. tuberculosis* antigens suggesting a long-lived memory T-cell response in these patients

and supporting the thesis that these antigens have the potential to evoke strong immunological memory,

The present study suggests that active tuberculosis patients elicit a very high antibody titre but a significantly low proliferative response to *M. tuberculosis* secreted antigens. The picture gets reversed after successful anti-tuberculosis treatment producing a better immune status in cured patients.

ACKNOWLEDGEMENT

Mrs. H. Uma was the recipient of Junior Research Fellowship from the Indian Council of Medical Research (ICMR), New Delhi. The authors thank Mrs. V. Shanthi for typing the manuscript.

REFERENCES

1. Dannenberg A.M., Jr. Immune mechanisms in the pathogenesis of pulmonary tuberculosis. *Rev. Infect Dis* 1989, 11 (suppl 2), S369.
2. Brennan P.J. Structure of mycobacteria recent developments in defining cell wall carbohydrates and proteins. *Rev. Infect. Dis* 1989, 11 (Suppl. 2), S420.
3. Hubbard R.D., Flory C.M. Collins F.M. Immunisation of mice with mycobacterial culture filtrate proteins. *Clin Exp immunol* 1991, 87, 94.
4. Grange J.M. The humoral immune response in tuberculosis; its nature, biological role and diagnostic usefulness. *Adv. Tuberc. Res,* 1984, 21, 1.
5. Emmrich F. Thole J. Van Embden J., Kaufmann S.H.E. *A* recombinant 64 kDa of *M. bovis* Bacillus Calmette-Guerin specifically stimulated human T4 clones reactive to mycobacterial antigens,. *J. Exp. Med,* 1986, 163, 10224.
6. Kleinhenz M.E., Eliner J.J.. Antigen responsiveness during tuberculosis : regulatory interactions of T-cell subpopulations and adherent cells. *J. Lab. Clin. Med* 1987, 110, 31.
7. Collins F.M. Pulmonary tuberculosis : the immunology of chronic infection. In : Vaccines and Immunotherapy. *S.J. Crys* (ed). 1991, Permagon Press New York.
8. Havlir D.V., Wallis R.S., Boom W.H. Daniel T.M.. Humain immune response to *M. tuberculosis* antigens. *Infect Immun* 1991, 59, 665.
9. Surcel M. Blomberg M.T., Paulie S., Andersson G. Moreno C., Pasvol G. Ivanyli T. Thl/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Immunology* 1994, 81, 171.
10. Torres M., Sampeiro P.M., Zamudio L.J. Teran-L. Camarena A., Quezada R., Ramos E, Sada E. Comparison of the immune response against M. tuberculosis antigens between a group of patients with active pulmonary tuberculosis and healthy household contacts. *Clin Exp. Immunol* 1994, 96, 75.
11. Boesen H, Jensen B.N. Wicke T., Andersen P. Human T-cell responses to secreted antigen fractions of *M. tuberculosis. Infect Immun.* 1995, 63, 1491.
12. Munk M.E. Emoto M. Functions of T-cell subsets and cytokines in mycobacterial infections. *Eur Respir J. Suppl.* 1995, 20, 668s-675s.
13. Boyum A. Separation of leukocytes from blood and bone-marrow. *Scand J Clin Lab Invest* 1968, 21, 31.
14. Selvaraj P., Uma H., Reetha A.M., Xavier T., Prabhakar R. Narayana P.R. Influence of HLA-DR2 phenotype on immunity and lymphocyte response to *M. tuberculosis* culture filtrate antigens in pulmonary tuberculosis. *Indian J Med Res* 1998, 107, 208.
15. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680.
16. Towbin H., Staehelin T. Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets, procedure and some applications. *Proc. Natl Acad Sci USA.* 1979, 76, 4350.
17. Paphia S.S., Wentzed J., Behjati F. Human leukocyte antigens and circulating immunoglobulin levels in Indian patients with pulmonary tuberculosis. *Tubercle* 1986, 66, 25.
18. Andersen P., Askgaard D. Ljungqvist J., Heron I. Proteins released from *M. tuberculosis* during growth. *Infect Immun* 1991, 59, 1905.
19. Nagai S., Wiker H.G., Harboe M., Kinomoto M. Isolation and partial characterisation of major protein antigens in the culture fluid of *M. tuberculosis. Infect immun* 1991, 59, 372.
20. Drowart A., Huygen K., De Bruyn J, Yernault J.C., Farber CM., Van Vooren J.P. Antibody levels to whole culture filtrate and to purified P32 during treatment of smear positive tuberculosis. *Chest* 1991, 100, 685.
21. Pessolani M.C.V. Peraita J.M., Rumjanek F.D. Serology and leprosy; immunoassays

comparing immunoglobulin G antibody responses to 28-30 kilodalton proteins purified from *M. bovis* BCG. *J Clin Microbiol* 1991, 29, 2285

22. Pitchappan R.M., Brahmajothi V., Rajaram K., Subramanyam P.T., Balakrishnan K., Muthuveeratlakshmi R. Spectrum of immune reactivity to mycobacterial (BCG) antigens in healthy hospital contacts in south India. Tubercle 1991, 72, 133.
23. Romagnani S. Lymphokine production by human T. cells in disease states. Ann Rev *Immunol* 1994, 12, 227.
24. Rossi G.A., Balbi G., Manta F. Evidence for selective presence of PPD-specific T-lymphocytes at site of inflammation in the early phase of the infection. *Am Rev Respir Dis* 1987, 136, 575.