

## Lymphocytotoxic antibodies & immunity in pulmonary tuberculosis

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To understand whether the presence of cold reactive lymphocytotoxic antibodies (LCA) (reactive at 15°C) in the system has any effect on immunity to tuberculosis lymphocytotoxic antibodies to adherent cells (enriched-B cells) and non-adherent cells were studied in active-TB (n=42) and inactive-TB (cured) patients (n=49) and healthy controls (n=32). The plasma samples of inactive-TB patients showed higher percentage of positivity for lymphocytotoxic antibodies (36.7%) than the active-TB patients (21.4%) and control subjects (18.8%). No significant difference on antibody and lymphocyte response against *Mycobacterium tuberculosis* culture filtrate antigens was observed between LCA positive and LCA negative active-TB patients and normal healthy controls. Further, determination of HLA-DR phenotype of the patients and control subjects showed that individuals positive for lymphocytotoxic antibodies were more among HLA-DR2 positive and DR7 positive active-TB patients and control subjects than non-DR2 and non-DR7 subjects. The present study suggests that the cold reactive lymphocytotoxic antibodies may be against B-lymphocytes and persistent for a longer time. HLA-DR2 and -DR7 may be associated with the occurrence of LCA activity. Further, the presence of LCA has no immunoregulatory role on immunity to tuberculosis.

**Key words** HLA-DR - lymphocyte response - lymphocytotoxic antibodies - *Mycobacterium tuberculosis* - pulmonary-TB

Lymphocytotoxins or lymphocytotoxic antibodies (LCA) are complement-binding antibodies that can occur in healthy individuals following viral or bacterial infections and after immunization or vaccination<sup>1</sup>. These antibodies have been shown in the sera of patients suffering from viral diseases<sup>2</sup> and also occur in several autoimmune diseases, malignancies and immunological diseases in which viral infection is implicated<sup>3-6</sup>. These lymphocytotoxic antibodies include both cold-reacting (IgM; react at 15°C) and warm reacting-antibodies (IgG; react at 37°C). Cold-reactive lymphocytotoxic antibodies have been found in non-viral infectious diseases such as tuberculosis<sup>1,7</sup> and leprosy<sup>8-11</sup>. Warm reacting antibodies have been shown to react with the major subsets of T-cells<sup>4,6</sup>. Lymphocytotoxic antibodies directed towards

specific T cell subsets have been reported in systemic lupus erythematosus (SLE)<sup>12</sup>. LCA activity in a number of auto-immune conditions, especially SLE and rheumatoid arthritis has been reported to be directed against T-lymphocyte subpopulation<sup>12,13</sup>. An immunoregulatory role for these antibodies has been postulated.

Although the biological stimulus for LCA production is not clearly understood, evidences indicate that anti-B cell LCAs are autoantibodies directed against self IgM<sup>14-16</sup>. The association of LCA with particular HLA-DR antigen has been studied in autoimmune diseases<sup>5,17,18</sup>. In mycobacterial diseases, most of the studies on LCA have been carried out in leprosy<sup>8-11,16</sup> with less attention being paid to tuberculosis<sup>1,7,19,20</sup>. Further, the

immunoregulatory role of these antibodies on the immunity to tuberculosis has not been explored. The present study was carried out to understand whether the presence of LCA in the system has an immunoregulatory effect on antibody and lymphocyte response to *Mycobacterium tuberculosis* culture filtrate antigen in patients with active and inactive tuberculosis and normal healthy control. Further, the influence of HLA-DR antigen on the occurrence of these antibodies in pulmonary tuberculosis has also been studied.

### Material & Methods

#### Study subjects:

(i) Active tuberculosis (ATB) patients - Patients attending the Tuberculosis Research Centre (TRC), Chennai, with respiratory symptoms and radiographic abnormalities suggestive of pulmonary TB were studied. These patients had sputum positive for *M. tuberculosis* by smear and culture. Blood samples were taken before the start of chemotherapy. Among the 42 ATB patients studied, there were 36 males and 6 females. The mean age (with SE) was  $40.1 \pm 6.7$  for males and  $26.8 \pm 2.2$  for females.

(ii) Inactive tuberculosis (ITB) patients (cured patients) - Patients classified as suffering from active pulmonary tuberculosis had received 10-15 yr earlier, a short course chemotherapy for 6-8 months at the TRC. At the time of blood sample collection all these cured patients were in the quiescent stage of the disease. Of the 49 cured patients studied, 42 were males and 7 were females. The mean age (with SE) was  $38 \pm 1.4$  for males and  $39.9 \pm 4.9$  for females.

(iii) Controls - Spouses of cured TB patients living together for 10-15 yr (family contacts; n=5) and clinicians, social workers, health visitors, laboratory volunteers and other staff (n=27) working at TRC for more than 3 years were studied. Among the 32 control subjects studied, 15 were males and 17 were females. The mean age (with SE) was  $40.8 \pm 2.3$  for males and  $37.9 \pm 2.2$  for females. All the family contacts and other control subjects were clinically normal at the time of blood sample collection.

The patients and spouses were not consanguineous to each other. The other control subjects were not related to any of the patients. The patients and the controls were a randomly selected population

belonging to the same ethnic origin. They were Tamil speaking south Indian population (belonging to different communities) living in and around Chennai.

This study was carried out during January to December 1995 at the Tuberculosis Research Centre, Chennai.

*Peripheral blood* : Peripheral blood mononuclear cells (PBMC) were separated from heparinised (20 units/ml) blood samples using Ficoll-hypaque density gradient centrifugation<sup>21</sup>. Plasma was used for antibody titre against *M. tuberculosis* and lymphocytotoxic antibodies. PBMC was used for lymphocytotoxic assay, HLA typing and lymphocyte response.

*Lymphocytotoxic antibody (lymphocytotoxin) assay*: The method described for serological determination of HLA has been used<sup>22</sup>. Briefly, plasma samples of patients as well as the controls were coated in Terasaki microtiter plates (Nunc, Denmark). Nylon wool column separated non-adherent (enriched T cell population) and adherent (enriched B cell population) cells (allogenic cells) were used as target cells. The non-adherent and adherent cells (2000 cells per well) were added to each well and incubated for 1 h at 15°C. Rabbit complement (5 µl/well) was added and incubated further for 2 h at 15°C. The dead cells were stained with 5 µl eosin (3%) and fixed in neutralised formalin. The plates were read in an inverted phase contrast microscope (Leitz, Portugal) at 160 x magnification. The cytotoxicity was assessed by scoring the reactions as 2, 4, 6 and 8 depending on the percentage of cytotoxicity as 11-20 per cent = 2; 21-40 per cent = 4; 41-80 per cent = 6; and 81-100 per cent = 8. A score of 6 and above was taken as a positive reaction for cytotoxic antibody. Plasma reacting against lymphocytes of three different subjects and above were considered as positive.

*HLA typing* : Serological determination of HLA-DR antigens was carried out in ATB and ITB patients and control subjects using nylon wool-adherent peripheral blood mononuclear cells (enriched B lymphocytes) by standard microlymphocytotoxicity assay<sup>22</sup>.

*Antibody and lymphocyte response to M. tuberculosis culture filtrate antigen* : *M. tuberculosis* culture filtrate antigen was prepared and used. The IgG antibody

titre against *M. tuberculosis* culture filtrate antigens was measured by ELISA. Lymphoproliferative response of the patients and control subjects to *M. tuberculosis* culture filtrate antigen (10 µg/ml) was studied<sup>23</sup>.

**Statistical analysis :** The frequency of HLA-DR antigens was calculated by direct allelic count. The level of significance of presence of lymphocytotoxic antibodies was ascertained by proportionality test and Student's 't' test for antibody titre and lymphocyte response. Results are expressed as arithmetic mean ± standard error (SE).

### Results

Lymphocytotoxic antibodies against allogenic lymphocytes were seen in active and cured pulmonary tuberculosis patients as well as normal healthy control subjects. A significantly higher percentage of ITB patients were positive for lymphocytotoxic antibodies than ATB patients and controls ( $P < 0.05$ ). Whereas, no difference was seen between ATB patients and controls (Table I). Plasma samples of active-TB, inactive-TB and control subjects reacted with nylon wool adherent cell population (enriched B-cell). These lymphocytotoxic antibodies were not against HLA antigens as judged by a panel of HLA typed cells (data not shown).

Presence of lymphocytotoxic antibodies in the system did not show any effect on antibody titre to *M. tuberculosis* antigens in LCA positive active-TB patients as well as control subjects than LCA negative patients and controls. However, a trend towards a decreased lymphocyte response (stimulation index)

to *M. tuberculosis* antigen (1 and 10 µg doses) was seen in LCA positive tuberculosis patients than LCA negative ATB patients (Table II). However, this trend was not significant. No such trend was seen in control subjects.

Among the subjects positive for lymphocytotoxins, an increased percentage of active TB patients with HLA-DR2 (55.6%) and HLA-DR7 (55.6%) were positive for lymphocytotoxic antibodies when compared to other DR antigens (Table III). However, in the DR6 positive active TB group, 44.4 per cent (4/9) of the patients were positive for lymphocytotoxic antibodies. Among the four patients three were DR6/DR7 positive heterozygotes.

### Discussion

In the present study, ATB and ITB patients as well as healthy controls showed lymphocytotoxic antibodies to allogenic mononuclear cells. These antibodies may be developed against self antigens (autoantigens) or antigens of *M. tuberculosis* origin, which may have structural similarity with self antigen.<sup>19</sup> During the active stage of the disease as well as during treatment with anti-TB drugs, the cellular components such as DNA and other nuclear/cellular proteins of the infected/lysed cells of the tissues, at the site of infection, may be exposed to the immune system which leads to autoantibody production. Under *in vivo* condition such antibodies may be reactive against the cell surface determinants of nucleated cells which may be involved in cell death either due to autoantibody and complement mediated cytotoxicity or cell necrosis or apoptosis.

**Table I.** Presence of lymphocytotoxic antibodies in control subjects, active-TB (ATB) and inactive-TB (ITB) patients to allogenic lymphocytes

Study subjects	No. of plasmatested	% of plasma positive for LCA	Positive for NA only	Positive for A only	Positive for NA and A
Controls	32	18.8 (n=6)	0	6	0
ATB	42	21.4 (n=9)	0	4	5
ITB	49	36.7 <sup>±</sup> (n=18)	0	17	1

LCA, lymphocytotoxic antibodies; NA, non-adherent cell population; A. adherent cell population

$P < 0.05^*$  as compared to controls and <sup>±</sup> ATB

**Table II.** Comparison of antibody titres and lymphocyte response to *M. tuberculosis* culture filtrate antigen in LCA positive and LCA negative patients and controls

Subjects	LCA positive					LCA negative			
	Antibody titre	Lymphocyte response in stimulation index, SI			Antibody titre	Lymphocyte response in stimulation index, SI			
		M.tb Ag. µg/ml				M.tb Ag. µg/ml			
		0.1	1.0	10.0		0.1	1.0	10.0	
Control (n=25)	400.0±182.4 (6)	4.2±1.1 (5)	12±6 (5)	15.9±5.7 (5)	451.6±142.4 (19)	3.8±0.9 (19)	8.1±1.6 (19)	18.6±3.2 (19)	
Active-TB (n=32)	2720.0±1065.3 (9)	1.4±1.2 (7)	2.2±0.5 (7)	4.0±0.7 (7)	2316.5±538.8 (23)	1.6±0.2 (23)	3.4±0.8 (23)	6.8±1.9 (23)	

LCA, lymphocytotoxic antibody; M.tb Ag, *M. tuberculosis* culture filtrate antigen

Figures in the parentheses represent the number of patients or control subjects studied

**Table III.** Influence of HLA-DR on lymphocytotoxic antibodies (LCA)

HLA-DR	Controls (n=6)	ATB (n=9)	ITB (n=18)
DR1+	1 (16.7)	0 (0)	2 (11.1)
DR1-	5 (83.3)	9 (100)	16 (88.9)
DR2+	3 (50)	5 (55.6)	8 (44.4)
DR2-	3 (50)	4 (44.6)	10 (55.6)
DR3+	1 (16.7)	0 (0)	6 (33.3)
DR3-	5 (83.3)	9 (100)	12 (66.7)
DR4+	1 (16.7)	1 (11.1)	5 (27.8)
DR4-	5 (83.3)	8 (88.9)	13 (72.2)
DR5+	1 (16.7)	2 (22.2)	4 (22.2)
DR5-	5 (83.3)	7 (77.8)	14 (77.8)
DR6+	1 (16.7)	4 (44.4)	2 (11.1)
DR6-	5 (83.3)	5 (55.6)	16 (88.8)
DR7+	3 (50)	5 (55.6)	5 (27.8)
DR7-	3 (50)	4 (44.4)	13 (72.2)
DR8+	0 (0)	1 (11.1)	0 (0)
DR8-	6 (100)	8 (88.9)	18 (100)
DR9+	0 (0)	0 (0)	0 (0)
DR9-	6 (100)	9 (100)	18 (100)
DR10+	1 (16.7)	0 (0)	4 (22.2)
DR10-	5 (83.3)	9 (100)	14 (77.8)

n, no. of individuals positive for lymphocytotoxic antibodies

Figures in parentheses are the percentage values

Most of the LCA positive plasma samples of the patients and control subjects reacted against adherent cells (enriched B cells). This shows that the antibodies reacting at 15°C may be against B-lymphocytes. Many studies carried out in leprosy patients revealed that most of these antibodies are cold reactive (react at 15°C) and developed against B-lymphocyte<sup>8-11,16</sup>. Further, it has been shown that these LCA have developed against surface IgM (immunoglobulin) of B-cells<sup>8-11,14,15</sup>.

A higher percentage of inactive-TB patients showed LCA activity against enriched B-cells. These ITB (quiescent) patients even 10 to 15 yr after treatment showed lymphocytotoxic antibodies. This shows the persistence of memory B-cells which may be involved in the production of lymphocytotoxic antibodies and it may persist for a longer time.

Presence of LCA in the system showed no dramatic effect on the immunity to tuberculosis. However, a trend towards a decreased lymphocyte response to *M. tuberculosis* antigens (10 µg) was seen in active-TB patients. This inhibitory/regulatory effect may be due to anti-idiotypic antibodies, which may be produced against autoantibodies. However, further studies are required to substantiate these preliminary data.

Among the LCA positive subjects (patients and controls) more number of HLA-DR2 and DR7

positive control subjects, active-TB and inactive-TB patients were seen. This suggests that HLA-DR2 and DR7 may enhance the production of lymphocytotoxic antibodies. An association between lymphocytotoxic antibodies and HLA-DR2 has also been reported in rheumatoid arthritis<sup>18</sup>. An increased anti-F (ab')<sub>2</sub> IgG auto-antibodies has been shown in HLA-DR2 positive pulmonary-TB patients as compared to -DR2 negative patients<sup>20</sup>. Our recent study of pulmonary tuberculosis patients revealed that HLA-DR2 is associated with increased antibody response and decreased lymphocyte response to *M. tuberculosis*<sup>23</sup>. Moreover, patients positive for HLA-DR2/DR7 heterozygous combination also showed a higher antibody production<sup>24</sup>. Since, HLA-DR2 is associated with far advanced smear positivity cases of pulmonary TB than with cases of minimal and moderate radiographic lung lesions<sup>25</sup> and extensive disease with drug failure cases<sup>26</sup>, augmentation of the production of lymphocytotoxic antibodies by HLA-DR2 and DR7 may further damage the cells and tissues of the lung which will be more detrimental to the host.

The present study suggests that lymphocytotoxic antibodies may occur during the active stage of tuberculosis. Persistence of LCA activity during the quiescent stage of the disease is evident. The occurrence of these antibodies may be associated with HLA-DR2 and DR7. LCA may play a role in the pathogenesis of tuberculosis.

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### References

1. Kreisler MJ, Hirata AA, Terasaki PI. Cytotoxins in disease 3-Antibodies against lymphocytes produced by vaccination. *Transplantation* 1970; 10 : 411-5.
2. Naito S, Mickey MR, Hirata A, Terasaki PI. Autolymphocytotoxins following immunization by pregnancy, transplantation and disease. *Tissue Antigens* 1971; 1 : 219-28.
3. Terasaki PI, Mottironi VD, Barnett EV. Cytotoxins in disease. Autocytotoxins in lupus. *N Engl J Med* 1970; 283 : 724-8.
4. Sakane T, Steinberg AD, Reeves JP, Green I. Studies of immune function of patients with systemic lupus erythematosus. T-cell subsets and antibodies to T-cell subsets. *J Clin Invest* 1979; 64 : 1260-9.
5. Taneja V, Mehra NK, Singh RR, Anand C, Malaviya AN. Occurrence of lymphocytotoxins in multi-case rheumatoid arthritis families : relation to HLA. *Clin Exp Immunol* 1991; 86 : 87-91.
6. Ozturk G, Terasaki PI. Non-HLA lymphocyte cytotoxins in various diseases. *Tissue Antigens* 1979; 14 : 52-8.
7. Kreisler M, Naito S, Terasaki PI. Cytotoxins in disease. V. Various diseases. *Transplant Proc* 1971; 3 : 112-4.
8. Nelson DS, Penrose JM, Waters MFR, Pearson JMH, Nelson M. Depressive effect of serum from patients with leprosy on mixed lymphocyte reactions: influence of anti-leprosy treatment. *Clin Exp Immunol* 1975; 22 : 385-92.
9. Serjeantson S, Dry P. Lymphocytotoxins in leprosy and in asymptomatic hepatitis B virus infection. *Clin Exp Immunol* 1980; 39 : 289-96.
10. Naik S, Kumar B, Kaur S, Sehgal S. Cold-reactive lymphocytotoxic antibodies in patients with tuberculoid and lepromatous leprosy. *Int J Lepr Other Mycobact Dis* 1987; 55 : 273-6.
11. Kaur I, Vaishnavi C, Kaur S, Sharma VK, Ganguly NK, Kohli M, *et al*. Lymphocytotoxins in leprosy. *Indian J Lepr* 1989; 61: 164-8.
12. Kumagai S, Steinberg AD, Green I. Antibodies to T-cells in patients with systemic lupus erythematosus can induce antibody-dependant cell-mediated cytotoxicity against human T-cells. *J Clin Invest* 1981; 67 : 605-14.
13. Utsinger PD, Yount WJ. Lymphopenia in Sjogren's syndrome with rheumatoid arthritis; relationship to lymphocytotoxic antibodies, cryoglobulinemia, and impaired mitogen responsiveness. *J Rheumatol* 1976; 3 : 355-66.
14. Ciccirelli JC, Chia D, Terasaki PI, Barnett EV, Shirahame S. Human IgM anti-IgM cytotoxin for B lymphocytes. *Tissue Antigens* 1980; 15 : 275-82.
15. Takahashi H, Terasaki PI, Iwaki T, Nakata S. Identification of surface IgM as the target antigen of cold lymphocytotoxins. *Tissue Antigens* 1980; 16 : 176-80.
16. Rasheed FN, Locniskar M, McCloskey DJ, Hasan RS, Chiang TJ, Rose P. *et al*. Serum lymphocytotoxic activity in leprosy. *Clin Exp Immunol* 1989; 76 : 391-7.
17. Wooley PH, Panayi GS, Batchelor JR. Lymphocytotoxins in rheumatoid arthritis: prevalence, lymphocyte specificity and HLA-DR antigens. *Ann Rheum Dis* 1981; 40 : 154-6.
18. Vischer TL, Jeannot M. Lymphocytotoxic antibodies in rheumatoid arthritis: relation to HLA-DR2 and rheumatoid factors. *Clin Exp Rheumatol* 1986; 4 : 139-42.
19. Shoenfeld Y, Isenberg DA. Mycobacteria and autoimmunity. *Immunol Today* 1988; 9 : 178-82.
20. Rajalingam R, Mehra NK, Chopra GS, Puri MM, Jain RC. Anti-IgG autoantibodies and possible immune regulatory mechanisms in patients with pulmonary tuberculosis. *Tuber Lung Dis* 1996; 77 : 502-9.

21. Boyum A. Isolation of mononuclear cells and granulocytes from human peripheral blood. *Scand J Clin Lab Invest* 1968; 21 (Suppl. 97) : 97-102.
22. Terasaki PI, McClelland JD. Microdroplet assay of human serum cytotoxin. *Nature* 1964; 204 : 998-9.
23. Selvaraj P, Uma H, Reetha AM, Xavier T, Prabhakar R, Narayanan PR. Influence of HLA-DR2 phenotype on humoral immunity and lymphocyte response to *Mycobacterium tuberculosis* culture filtrate antigens in pulmonary tuberculosis. *Indian J Med Res* 1998; 107 : 208-17.
24. Uma H. *Studies on the influence of human leucocyte antigens (HLA-DR & DQ) on immune responses in pulmonary tuberculosis*, Ph.D. thesis, Chennai : Tamil Nadu Dr M.G.R. Medical University; 1998.
25. Brahmajothi V, Pitchappan RM, Kakkanaiah VN, Sashidhar M, Rajaram K, Ramu S. *et al.* Association of pulmonary tuberculosis and HLA in south India. *Tubercle* 1991; 72 : 123-32.
26. Rajalingam R, Mehra NK, Jain RC, Myneedu VP, Pande JN. Polymerase chain reaction based sequence-specific oligonucleotide hybridisation analysis of HLA-class II antigens in pulmonary tuberculosis: Relevance to chemotherapy and disease severity. *J Infect Dis* 1996; 173 : 669-76.

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