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## HLA-DR phenotypes and IgG, IgA and IgM antibody responses to *Mycobacterium tuberculosis* culture filtrate and 30 kDa antigens in pulmonary tuberculosis

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The role of HLA-DR genetic make-up on the IgG, IgA and IgM antibody response to *Mycobacterium tuberculosis* culture filtrate and 30 kDa antigens was studied in pulmonary tuberculosis. The study was carried out in HLA-DR typed active pulmonary tuberculosis (ATB) patients ( $n = 37$ ), inactive (cured) pulmonary tuberculosis (ITB) patients ( $n = 79$ ) and normal healthy subjects (NHS;  $n = 46$ ). In ATB and ITB (cured) patients, IgG antibody (optical density at 490 nm for 1 : 3200 dilution) as measured by enzyme-linked immunosorbent assay was the predominant one than IgA and IgM antibodies. Increased IgG antibody titre to culture filtrate ( $P = 0.03$ ) and decreased titre to 30 kDa antigen were observed with HLA-DR1-positive ATB patients than non-DR1 (ATB) patients. Moreover, HLA-DR4- and HLA-DR6-positive ATB patients showed trends toward an increased IgG antibody response to 30 kDa antigen than HLA-DR4- and HLA-DR6-negative (ATB) patients respectively. Significantly increased IgA antibody to 30 kDa antigen was observed with HLA-DR1-positive ATB patients than non-DR1 patients ( $P = 0.03$ ). The study suggests that multiple HLA-DR molecules may regulate the IgG and IgA antibody responses to various proteins of *M. tuberculosis*. Moreover, HLA-DR phenotypes and increased IgG and IgA antibody titres may be useful to differentiate *M. tuberculosis*-infected subjects from normal subjects and cured patients with the same HLA-DR phenotypes or genetic make-up.

MYCOBACTERIAL infections are associated with the production of circulating antibodies and development of cell-mediated immune functions. Although antibody titres tend to be higher in tuberculosis patients than normal subjects, results have been disappointing for serodiagnosis of tuberculosis because of overlapping antibody response. Protein antigens of mycobacteria are characterized by cross-reactivity when analysed for antibodies obtained by experimental immunization or by those arising during natural mycobacterial infection. Species specificity is rare at the level of individual proteins, as would be expected of major proteins of functional significance<sup>1,2</sup>. Identification of antigens and their antigenic determinants is im-

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portant for an understanding of the interactions involved in the immune response to *Mycobacterium tuberculosis* and may be of potential value in the design of diagnostic reagents and subunit vaccines. In this context, the growing number of defined and available mycobacterial antigens now provide a rational basis for subunit vaccine design and development of specific diagnostic reagents<sup>3</sup>. Of the many *M. tuberculosis* antigens, antigen 85 complex (30–31 kDa), 65 kDa antigen, 38 kDa antigen, 16 kDa antigen and ESAT-6 are some which are well characterized for serodiagnosis as well as for subunit vaccine. Subunit vaccine approach has to rely on identification and application of protein antigens recognized by protective T-cells within HLA heterogenous population<sup>4</sup>. Recently, it has been shown that the memory T-cell responses to 85 kDa antigen complex, peptides of ESAT-6 and 65 kDa heat-shock protein are subject to effective host genetic regulation<sup>5</sup>. HLA restriction of CD8+ T-cell epitope of 85 complex of *M. tuberculosis*<sup>6,7</sup> and naturally derived Th1 cell epitopes of 85B (30 kDa) antigen of *M. tuberculosis* has been studied<sup>8</sup>. Earlier studies carried out in HLA and tuberculosis revealed an increased antibody response to 38 kDa antigen in patients with HLA-DR2 phenotype<sup>9</sup>. Further, it has been shown that HLA-DR15, a split of HLA-DR2 is associated with high levels of antibodies to 38 kDa antigen<sup>10</sup>. The influence of HLA-DR on humoral and lymphocyte response in pulmonary tuberculosis has also been studied by us earlier<sup>11,12</sup>. The present study was attempted to understand the regulatory role of polymorphic HLA-DR molecules on the IgG, IgA and IgM antibody responses to *M. tuberculosis* antigens, as well as to find out whether HLA-DR genetic make-up of the individuals is useful to differentiate *M. tuberculosis*-infected subjects (active TB patients) from the endemic normal subjects and cured patients by detecting IgG, IgA and IgM antibodies to culture filtrate antigen (CFA) and 30 kDa antigen.

Subjects included were 37 active pulmonary tuberculosis (ATB) patients, 79 inactive tuberculosis (ITB) patients (cured) and 46 normal healthy subjects (NHS). The mean age with standard error was  $38.5 \pm 1.9$ ;  $40.2 \pm 1.2$  and  $37.3 \pm 1.3$  yr respectively. ATB patients attending Tuberculosis Research Centre, Chennai with respiratory symptoms and radiographic abnormalities suggestive of pulmonary TB were studied. They were sputum-positive for *M. tuberculosis* by smear and culture. These patients were considered as the infected group. Blood samples were collected before the start of chemotherapy. ITB (cured patients) were selected from among subjects of an earlier chemotherapy study. Patients classified as suffering from ATB had received supervised short-course chemotherapy of 6–8 months duration and had been followed up for five years after treatment. At the time of blood-sample collection, all these cured patients were in the quiescent stage of the disease. NHS ( $n = 46$ ) consisted of laboratory volunteers ( $n = 32$ ) of our centre working for more than

three years and the patient contacts ( $n = 14$ ) (spouses of the patients) who were living together with the patients before, during and after treatment. The patients and NHS belonged to the same ethnic origin.

Twenty millilitres of peripheral blood was drawn in heparin (20 units/ml) from patients and NHS. The mononuclear cells were separated using ficoll hypaque density gradient centrifugation as described by Boyum<sup>13</sup>. Plasma was aspirated and stored at  $-20^{\circ}\text{C}$  until use. T- and B-lymphocytes were separated from peripheral blood mononuclear cells using nylon wool column and adherent cell population (enriched B-lymphocytes) was used for HLA-DR typing using two-stage microlymphocytotoxicity assay<sup>14</sup>. Commercial sources of antisera (Biotest, Frankfurt, Germany) were used for HLA-DR typing and at least three antisera were used for each specificity studied<sup>15</sup>.

CFA and 30 kDa antigen were prepared as described earlier<sup>16,17</sup>. Briefly, *M. tuberculosis* H37Rv was grown in Sauton's liquid medium for 6 weeks as a surface pellicle. Bacilli were removed by centrifugation and the culture supernatant was filtered through Seitz filter. The proteins in the culture filtrate were precipitated with 90% ammonium sulphate saturation. The final precipitate was dissolved and dialysed against PBS (0.1 M, pH 7.2) extensively. The antigen was aliquoted and stored at  $-70^{\circ}\text{C}$  in the presence of sodium azide and the protease inhibitor phenylmethyl sulfonyl fluoride<sup>16</sup>.

Two-step purification was carried out for the separation of 30 kDa antigen (85B) from CFA<sup>17</sup>. The first-step purification included anion exchange chromatography (QAE-Sepharose). Tris-HCl 0.05 M, pH 8.1-buffer was used as the starting buffer and a linear gradient of 0–100% 0.05 Tris-HCl, pH 8.1 with 1.0 M sodium chloride was used for elution. The run was carried in the High Performance Liquid Chromatography system (Millennium V2.00, Waters, USA). The Ag85 complex containing fractions were pooled. Second-step purification of antigen A, B and C components from the Ag85 complex was done by passing it through the hydrophobic interaction chromatography column, namely the phenyl sepharose HP column using three different buffers: buffer A consisting of 0.01 M  $\text{NaH}_2\text{PO}_4$ , pH 6.8 for washing; buffer B, 0.01 M Tris-glycine pH 8.0 for binding and buffer C, 0.01 M Tris-glycine with 50% ethylene glycol, pH 8.9 for elution. The fraction containing antigen 85 B alone was used to sensitize ELISA plates at a concentration of  $1 \mu\text{g}/\text{ml}$ .

IgG, IgA and IgM antibody levels against CFA and 30 kDa antigen of *M. tuberculosis* H37Rv were estimated in the plasma of HLA-DR typed ATB patients, ITB (cured) patients and NHS.

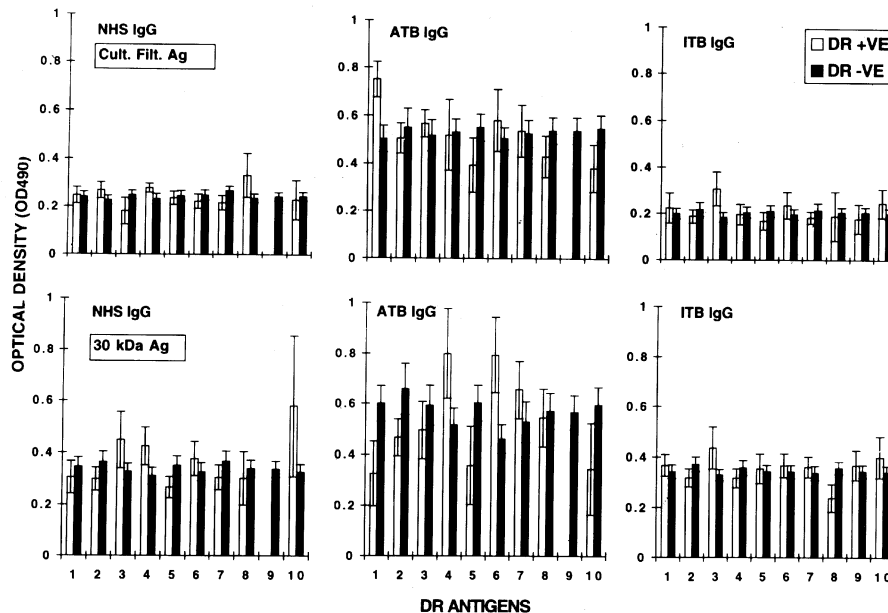
ELISA plates (Nunc Maxisorp Certified, flat-bottomed) were coated with an optimal concentration of purified antigens of *M. tuberculosis* in 0.06 M carbonate buffer, pH 9.6 and incubated overnight at  $4^{\circ}\text{C}$ . The plates were washed four times with PBS containing 0.1% Tween 20

(PBST) using the automatic ELISA washer (Organon Teknika, Austria). The non-specific sites in the wells were blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C. After four washes with PBST, the plates were incubated with sera from patients and normals (1:3200 dilution). The 1:3200 dilution was selected based on the previous experiments. The plates were washed after 1 h incubation at 37°C and further incubated with anti-human IgG, IgA or IgM peroxidase conjugate (Jackson, USA) in PBST containing 1% BSA. After 1 h of incubation at 37°C, the plates were washed and colour developed by the addition of 100 µl of substrate (50 ml of phosphate-citrate buffer, containing 20 mg of ortho-phenylenediamine and 30 µl of hydrogen peroxide) to each well. The reaction was arrested by adding 50 µl of 8N H<sub>2</sub>SO<sub>4</sub>. The plates were read using Spectramax ELISA reader (Molecular Devices, USA) at 490 nm wavelength. Pooled TB plasma and PBST

buffer were used as the positive and negative controls respectively, in each plate. The mean absorbance and standard error (SE) of the normal were calculated.

The frequencies of HLA-DR antigens in patients and controls were determined by direct count. The results on antibody titre (OD<sub>490</sub> at 1:3200 dilution) are expressed as mean ± SE. Student's *t* test was applied to see the significance of the data. *P* values less than 0.05 are considered as significant.

The antibody response to *M. tuberculosis* CFA and 30 kDa antigen as measured by the optical density (OD<sub>490</sub>) at 1 in 3200 dilution, was higher in ATB patients than ITB (cured) patients and NHS. The IgG antibody response to CFA and 30 kDa antigen was higher in ATB patients compared to cured patients and NHS. Moreover, IgG antibody titre was higher than IgA and IgM antibodies (data not shown).



**Figure 1.** HLA-DR phenotypes and IgG antibody response (optical density at 490 nm) to *M. tuberculosis* culture filtrate and 30 kDa antigen in normal healthy subjects (NHS), active pulmonary tuberculosis (ATB) and inactive pulmonary tuberculosis (ITB) patients. The results are expressed as arithmetic mean ± standard error

	NHS (n)	ATB (n)	ITB (n)		NHS (n)	ATB (n)	ITB (n)
HLA-DR1-positive	10	4	12	Non-DR1	36	33	67
HLA-DR2-positive	18	17	38	Non-DR2	28	20	41
HLA-DR3-positive	4	9	12	Non-DR3	42	28	67
HLA-DR4-positive	11	7	23	Non-DR4	35	30	56
HLA-DR5-positive	11	5	15	Non-DR5	35	32	64
HLA-DR6-positive	10	12	10	Non-DR6	36	25	69
HLA-DR7-positive	22	12	28	Non-DR7	24	25	51
HLA-DR8-positive	3	3	8	Non-DR8	43	34	71
HLA-DR9-positive	ND	4	6	Non-DR9	ND	ND	73
HLA-DR10-positive	ND	ND	6	Non-DR10	ND	33	73

ATB: HLA-DR1-positive vs HLA-DR1-negative: IgG antibody to CFA *P* = 0.03; *n*, Number of individuals studied in each group, ND, Not determined.

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**Table 1.** IgA antibody titre (optical density at 490 nm) to CFA and 30 kDa antigen in selected HLA-DR phenotypes of NHS, ATB and ITB patients

HLA-DR phenotype	CFA			30 kDa antigen		
	NHS	ATB	ITB	NHS	ATB	ITB
DR1-positive	0.199 ± 0.062 (10)	0.599 ± 0.185 (4)	0.276 ± 0.044 (12)	0.173 ± 0.033 (10)	1.018* ± 0.179 (4)	0.390 ± 0.045 (12)
DR1-negative	0.232 ± 0.030 (36)	0.344 ± 0.045 (33)	0.333 ± 0.026 (67)	0.269 ± 0.038 (36)	0.373* ± 0.040 (33)	0.405 ± 0.038 (67)
DR4-positive	0.293 ± 0.079 (11)	0.414 ± 0.074 (7)	0.297 ± 0.040 (23)	0.300 ± 0.041 (11)	0.408 ± 0.093 (7)	0.332 ± 0.047 (23)
DR4-negative	0.202 ± 0.023 (35)	0.362 ± 0.054 (30)	0.336 ± 0.028 (56)	0.231 ± 0.039 (35)	0.450 ± 0.061 (30)	0.432 ± 0.042 (56)
DR6-positive	0.240 ± 0.038 (10)	0.312 ± 0.072 (12)	0.290 ± 0.033 (10)	0.373 ± 0.104 (10)	0.336 ± 0.075 (12)	0.513 ± 0.128 (10)
DR6-negative	0.220 ± 0.033 (36)	0.396 ± 0.058 (25)	0.312 ± 0.025 (69)	0.213 ± 0.026 (36)	0.494 ± 0.066 (25)	0.387 ± 0.033 (69)

Numbers in parentheses represent the individuals studied in each group. \* $P = 0.03$ .

**Table 2.** IgM antibody titre (optical density at 490 nm) to CFA and 30 kDa antigen in selected HLA-DR phenotypes of NHS, ATB and ITB patients

HLA-DR phenotype	CFA			30 kDa antigen		
	NHS	ATB	ITB	NHS	ATB	ITB
DR1-positive	0.114 ± 0.045	0.734 ± 0.315	0.424 ± 0.126	0.264 ± 0.048	0.537 ± 0.172	0.402 ± 0.064
DR1-negative	0.232 ± 0.031	0.250 ± 0.054	0.290 ± 0.029	0.402 ± 0.043	0.342 ± 0.045	0.340 ± 0.021
DR4-positive	0.206 ± 0.033	0.150 ± 0.045	0.351 ± 0.077	0.401 ± 0.077	0.231 ± 0.069	0.366 ± 0.034
DR4-negative	0.206 ± 0.034	0.338 ± 0.075	0.294 ± 0.031	0.363 ± 0.041	0.394 ± 0.051	0.338 ± 0.024
DR6-positive	0.285 ± 0.080	0.239 ± 0.065	0.272 ± 0.044	0.448 ± 0.087	0.396 ± 0.095	0.352 ± 0.055
DR6-negative	0.184 ± 0.026	0.332 ± 0.087	0.316 ± 0.035	0.351 ± 0.039	0.347 ± 0.048	0.349 ± 0.022

Number of individuals studied in each group is similar to that in Table 1.

An increased IgG antibody response to CFA was observed in HLA-DR1-positive ATB patients than HLA-DR1-negative ATB patients ( $P = 0.03$ ), whereas a trend towards a decreased IgG antibody titre to 30 kDa antigen was observed in HLA-DR1-positive ATB patients than HLA-DR1-negative ATB patients. Such a difference was not observed in NHS or cured patients to CFA and 30 kDa antigen (Figure 1). Among the ATB patients, HLA-DR4- and HLA-DR6-positive patients showed trends toward an increased IgG antibody response to 30 kDa antigen when compared to HLA-DR4- and HLA-DR6-negative patients respectively.

HLA-DR1-positive ATB patients showed a significantly increased IgA antibody response to 30 kDa antigen than DR1-negative (non-DR1) ATB patients ( $P = 0.03$ ). Similar trend was also observed with CFA. No marked change in the IgA antibody titre was observed in HLA-DR4- and HLA-DR6-positive ATB patients compared to HLA-DR4- and HLA-DR6-negative patients (Table 1). The IgM antibody responses to various *M. tuberculosis* antigens studied were not influenced by HLA-DR phenotypes (Table 2).

In the present study, higher IgG antibody response to CFA and 30 kDa antigen of *M. tuberculosis* was observed in ATB patients than ITB (cured) patients and NHS. This suggests that IgG antibody is the predominant antibody

which may be produced during the active stage of the disease and may persist for a longer time even after cure. It has been suggested that the antibody levels are more closely related to the disease activity and anti-TB treatment<sup>18,19</sup>. Moreover, the IgG, IgA and IgM antibody responses of cured patients (cured ten years back) were comparable to those of NHS, which reflects a picture similar to that of NHS.

HLA-DR1-positive ATB patients exhibited a significantly higher IgG antibody titre to CFA and IgA antibody titre to 30 kDa antigen than HLA-DR1-negative ATB patients. This suggests that HLA-DR1 may probably augment IgG and IgA antibody responses to *M. tuberculosis* antigens during the active stage of tuberculosis, probably through a shift from Th1 (associated with protective immunity) to Th2 type (associated with humoral immunity) of response. Moreover, HLA-DR4- and HLA-DR6-positive ATB patients showed a trend towards a higher IgG antibody titre to 30 kDa antigen than HLA-DR4-negative and HLA-DR6-negative ATB patients. This suggests that multiple major histocompatibility complex molecules may regulate the humoral immune responses to the proteins of *M. tuberculosis* antigens during the active stage of the disease. Multiple HLA-DR molecules have been shown to be associated in inducing protective Th1 res-

ponse to 18, 28 and 65 kDa proteins of *M. tuberculosis*<sup>20</sup>. This may be due to the recognition of different epitopes of the *M. tuberculosis* antigens by different HLA-DR molecules in association with T-cell receptor. Further, the present study suggests that HLA-DR1, HLA-DR4 and HLA-DR6 phenotypes may be useful to differentiate *M. tuberculosis*-infected subjects from NHS and cured patients with HLA-DR1, HLA-DR4 and HLA-DR6 phenotypes based on the high IgG and IgA antibodies to *M. tuberculosis* CFA and 30 kDa antigen. Moreover, IgG and IgA antibody responses to a battery of *M. tuberculosis* antigens may throw more light on HLA-DR phenotype-based differentiation of *M. tuberculosis*-infected individuals from NHS and cured patients in a genetically heterogenous population.

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## Haemoglobinopathies – thalassaemias and abnormal haemoglobins in eastern Uttar Pradesh and adjoining districts of neighbouring states

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**The haemoglobinopathies – thalassaemias and abnormal haemoglobins – constitute a major burden of genetic diseases in India. Our study, based on index cases from 120 families detected between May 1999 and May 2003, highlights the ethnic distribution of haemoglobinopathies in regions in and around Varanasi comprising 8–10 districts of eastern Uttar Pradesh and**

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