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IMMUNE RESPONSE TO *MYCOBACTERIUM. TUBERCULOSIS* CULTURE FILTRATE ANTIGEN IN CURED SPINAL TUBERCULOSIS PATIENTS AND THEIR SPOUSES

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Summary :Humoral and cell mediated immune responses were studied in cured spinal tuberculosis patients and their spouses to understand immunit to tuberculosis in cured patients. Antibody litre and immune complex levels were measured and lymphocyte response to *Mycobaclerinm* ***tuberculosis*** culture filtrate antigen was observed in cured spinal tuberculosis patients (n = 3 0) and their spouses (n =27). A trend towards increased antibody litre was seen in cured patients as compared to their spouses. Significantly increased circulating immune complex levels, as measured by PEG OD280 (polyethylene glycol optical density 280) were seen in the contacts compared to cured patients. And a trend towards increased lymphocyte response to *Mtuberculosis* culture filtrate antigen was seen with different antigen concentrations (0.1,1 and 10 µg /ml). Moreover, the effect of active-pulmonary-Tuberculosis (AT B) plasma taken from 1ILA-DR2 positive and DR2 negative patients on lymphocyte response of the cured patients showed no dramatic immunomodulatory effect in the lymphocyte response when treated with DR2 positive or DR2 negative plasma. The study suggests that the memory response lo *Mtuberculosis* is well maintained even after 10-15 years of treatment

Keywords : Antibody litre, immune complex, lymphocyte response, spinal tuberculosis

INTRODUCTION

Tuberculosis is a chronic infectious disease regulated almost entirely by the CMI response of the host against *Mtuberculosis.* Our earlier studies in active pulmonary tuberculosis and cured pulmonary tuberculosis revealed that during the active stage the humoral immune response is augmented but the antigen induced lymphocyte response *(in vitro* correlate of CMI response) is suppressed'. And, that when the disease is cured, the antibody response declines and lymphocyte response to antigens increases to the same level as that found in control subjects. Moreover, in the treated patients (cured) the memory response to *M.tuberculosis* antigen is retained.

Our studies also revealed that HLA-DR2 is associated with susceptibility to pulmonary tuberculosis and increased antibody litre to *M tuberculosis* antigens[2,3]. Moreover, a low spontaneous lymphoproliferative response (without antigen stimulation) was also seen in HLA-DR2 positive active pulmonary tuberculosis patients compared with HLA-DR2 negative patients' Since, a low spontaneous lymphocyte response was seen in HLA-DR2 positive active tuberculosis patients, (he present study was designed to study if the plasma of the DR2 positive patients had any immunomodulatory effect on the lymphocyte response of cured spinal tuberculosis patients. The immune status of the cured patients was also studied.

MATERIAL AND METHODS

Study Subjects

(i) *Spinal tuberculosis patients* : Patients (n=30) enrolled in another study[4] who had active spinal tuberculosis involving any vertebral body from the first thoracic to the first sacral inclusive were eligible for the study. Patients were ineligible in case of (i) paralysis of lower limbs, (ii) serious extra-spinal disease (tuberculous or non-tuberculous), (iii) a history of previous chemotherapy for 12 months or more and (iv) major surgery undergone earlier. Pre-treatment investigations included (a) complete clinical examination, (b) radiographs of the spine and chest, (c) culture of pus from any abscess, sinus, if present

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as well as 3 sputum specimens from radiologically proven pulmonary tuberculosis patients and (d) sensitivity tests to Isoniazid and Rifampicin on positive cultures of *M tuberculosis* The patients had been treated with either radical surgery involving bone grafting or short course chemotherapy (6-9 months), 10-15 years ago. Of the 30 patients, 16 were males and 14 were females. The mean age of the patients ±SE was 42.93 ± 2.86 years.

(ii) *Controls.* Spouses of the study patients included in the group(n =27) were clinically normal at the time of blood specimen collection. The patients and their spouses were not consanguinous but had the same ethnic origin. Of the 27 controls, 12 were males and 15 were females. The mean age of the controls was 42.62± 2.68 years.

ELISA Test

M tuberculosis H37Rv culture filtrate antigen 5 µg in 100 µl in carbonate buffer (pH 9.6) was coated on 96 well round bottomed polyvinyl plates (Costar, Cambridge, MA, USA). The plates were left overnight at 4°C. Each well was then washed thrice with PBS containing 0.1 % Tween(PBST) and blocked with 1% BSA at 37°C for I hour. One 100 µ1 of the patient plasma was added in 1-5 dilution in duplicates and then serially diluted to 1:5- 1:20 dilution and incubated at 37°C for 1 hour after which the plates were washed again and 100 µ1 rabbit anti-human 1g(i peroxidase conjugate (1:500 dilution) (Bangalore Cicnei Pvt. 1 Id., Bangalore, India) was added in each well After each incubation at 37°C for 1 hour, the plates were washed with PBST and orthophenylenediamine (OPD 0.5 ing/ml) (Sigma Chemical Co., St. Louis, USA) with 0.1 % hydrogen peroxide was added as substrate. The reaction was stopped by adding 50 µ1 of 8N sulphuric acid and the colour was read at 490nm in a spectrophotometer (Molecular Devices, USA). One row of blanks was included in each test, fwice the mean optical density (OD) of the blank value was taken as positive for each specimen.

PEG Precipitation of Immune Complexes

The plasma specimens were cenrifuged at 16,000 rpm in a inicrocentrifuge (Lppcndorf, Netheler, GmbH, Germany) for 10 minutes to eliminate the cryoglobulins and other precipitated proteins which are involved in coagulation Plasma specimens were then precipitated with PEG(Polyethy1ene glycol -600)[5] 500µl of serum was mixed with an equal volume of 7% PUG and incubated at 4°C overnight. The precipitated material was centrifugcd in a microcentrifuge, the pellet was washed twice with 3.5% PKG, and then reconstituted to the original volume in HBSS (Hank's balanced salt solution). Protein content was estimated by reading the absorbance at 280nm in a spectrophotometer (SpectraMAX 250, Moleculai Devices, USA)

LymphoprolifemtiveAssay

For this, 0.1 X 10^6 peripheral blood mononuclear cells (separated by Ficoll-hypaque density gradient method) of the study patients and controls were cultured in a 96-well U-bottomed tissue culture plate in a total volume of 200 µ1 in each well in RPMI medium (Sigma Chemical Co., USA) supplemented with 2mM F-glutamine (Flow Laboratories, Irvine, Scotland, UK), Gentamycin (l0mg/ml), (M.A. Bio Products, Walkersville, Maryland, USA) and 10% autologous plasma. The cultures were stimulated with (i) three different concentrations of *M tuberculosis* H37Rv culture filtrate antigen (0.1 µg/ml. 1µg/ml and 10 µg/ml), (ii) the above concentrations of *Mtuberculosis* IH37Rv culture filtrate antigen and HLA-DR2 positive plasma or HLA-DR2 negative plasma of active pulmonary tuberculosis patients and (iii) only HLA-DR2 positive or HLA-DR2 negative plasma of active pulmonary tuberculosis patients. Cells cultured in medium only, without any stimulation, served as control. The culture was set up at least in triplicates. The cultures were maintained for six days, at 37°C and 5% carbon dioxide. Sixteen hours before harvesting, the cells were pulsed with lµCi of tritiatcd thymidine. The cells were harvested onto glass fibre filter discs. The lymphocyte response to tuberculosis antigen was measured by ti ilium labeled thymidine uptake by the cells in a Beta scintillation counter (LKB, Wallac Oy. Turku Finland). The mean cpm value of the triplicate cultures was calculated and the stimulation index was derived using the formula :

$$SI = \frac{\text{(Mean cpm of the test)}}{\text{mean cpm of the control)}}$$

RESULTS

The ELISA results *of M.tuberculosis* H37Rv culture filtrate antigen showed an increase in the antibody titre in spinal tuberculosis patients as compared to their contacts. This increase was, however, not significant (P>0.05). There was a significantly increased level of immune complexes in the control group as compared to the patient group (0.69 ± 0.06 vs 0.313 + 0.05); (PO.05) (Table 1).

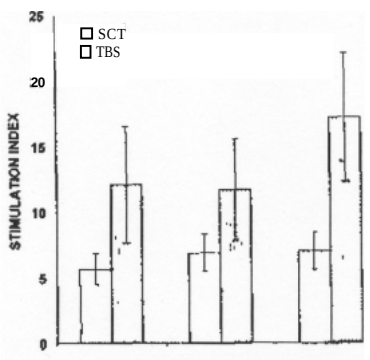
Table 1. IgG Antibody titre and immune complex levels in cured spinal tuberculosis patients and their spouses(fami1y contacts)

Subjects	Antibody titre	Immune complex level
Contacts (n=27)	86.8±26.4	0.69±0.06
Patients (n=30)	188.2± 56.9	0.31±0.05*

Theresults arc expressed as arithmetic mean± SE

*P<0.05

The results of lymphoproliferative assay of



M. tuberculosis culture filtrate antigens in the three

Fig. 1 : Peripheral blood hmphocyle response to various coiiccntiatmns of *M tuberculosis* culture filtrate antigen in stud) patients and their contacts F.ach bar represents Mean ±SE Cured Spinal M. tuberculosis Cases (TBS) 30 Spouse Contacts as Controls (SCT) 27

concentrations used showed a trend towards increased proliferation in patients as compared to their contacts and there was an increasing proliferation with increasing concentrations of antigen used. At 10 µg /ml concentration, the proliferation in the patients almost reached significance when compared to the contacts (Fig. 1).

When proliferation was assayed in the presence or absence of HLA-DR2 plasma alone, there was no

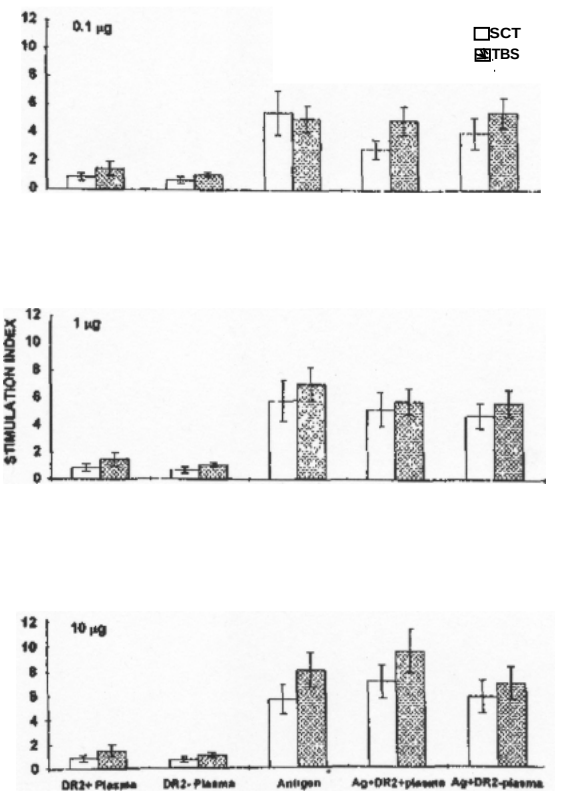


Fig 2 : Effect of HEA-DR2 positive and HLA-DR2 negative plasma of active study-TB patients on lymphocyte response in patients and their contacts.

Each bar represents Mean ±SE
cured Spinal Tuberculosis Cases (FBS) 30
Spouse Contacts as Controls (SCT) 27

significant difference in patients and the controls but there was a trend towards increased proliferation in the patients in the presence of DR2 positive plasma, especially at higher concentrations of antigen (10 u.g /ml) (Fig. 2)

DISCUSSION

A trend towards increased IgG antibody titre to *M tuberculosis* culture filtrate antigen was seen in the cured patients of tuberculous spine (TBS) as compared to their spouses(SCT). Our earlier study had revealed an increased antibody titre in the cured pulmonary tuberculosis patients'. In pulmonary tuberculosis, there is heavy bacillary (antigenic) load which may be responsible for triggering a B-cell response, whereas in spinal tuberculosis the disease is limited to spine and is paucibacillary. However, some cases were also positive for radiologic abnormalies with a presumably higher bacillary load. This means that IgG antibodies response either to low or medium levels of bacillary load induces a good antibody response as well as memory B-cell response. This results in persistence of high IgG antibody levels even in the cured.

Even though there is increased IgG antibody titre in the cured spinal tuberculosis patients, the circulating immune complex level was found to be decreased in patients compared to their spouses (contacts). It has been suggested that in pulmonary tuberculosis the reactive patients (increased CMI response) with localised lesions have little free antibodies and no or low level of immune complexes in serum. Moreover, in patients with inactive disease, the proportion of immune complexes is lower[6]. In another study, a significantly higher level of CICs has been shown in the control group[7].

The increased antibody titre in the cured patients may be mainly due to a memory response showing persistent antibody titre and increased IgG antibody response. Antigen-antibody complexes may cause granuloma formation, a phenomenon characteristic of mycobacterial infection. The presence of immune complexes after tuberculosis infection may play an immunopathologic role. The increased CIC levels found in the contacts may be due to exposure to mycobacterial antigens or autologous antigens altered by the infective organisms or inflammatory process or host immunoglobulins, like the rheumatoid factor[8].

Immunity in tuberculosis being a purely cell-mediated defence', lymphoproliferative response to antigen or mitogen stimulation has been widely used as *in vitro* correlate of cell mediated immunity[10,11]. The increased lymphocyte response to different concentrations *of M. tuberculosis* antigens, found in the cured spinal tuberculosis patients may be due to the presence of sensitized T-cells to *M.tubercu1osis* antigens, suggesting a prolonged memory T-cell response. Our earlier study had also revealed a similar picture in cured pulmonary tuberculosis patients'.

Our earlier study had revealed that HLA-DR2 is associated with susceptibility to pulmonary tuberculosis'. Moreover, DR-2 antigen is also associated with increased antibody titre and decreased spontaneous lymphoproliferative response, which is an *in vitro* correlate of CMI response[3]. In the present study, cultures stimulated with plasma samples of active tuberculosis patients positive for HLA-DR2 did not show any significant modulatory effect (suppressed or enhanced response) on lymphocytes either with or without *M.tuberculosis* culture filtrate antigen stimulation, in cured spinal tuberculosis patients. This suggests that the suppressed spontaneous lymphocyte response may be due to altered cytokine factors(Th1 to Th2) or other factors released during the active stage of the disease.

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