Association of increased mycobacterium growth inhibitory factor with antituberculous immunity

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ABSTRACT: Cell-mediated immune mechanisms (CMI) were studied in 51 patients of pulmonary tuberculosis to evaluate the role of mycobacterium growth inhibitory factor in prognosis of the infection, before and after the administration of anti-tubercular drugs. Twenty five Mantoux negative individuals who were subsequently bacille Calmette-Guérin (BCG) vaccinated and 25 Mantoux positive, non-tuberculous controls were included in the study. Their clinical assessment was compared with skin sensitivity (Mantoux); lymphocyte transformation (LT) after stimulation with phytohaemagglutinin (PHA) and purified protein derivative (PPD); macrophage migration inhibitory factor (MIF), mycobacteria growth inhibitory factor (Myco IF) and listerial growth inhibitory factor (List IF). The tests were carried out at the beginning of the treatment and at intervals of three months, extending to one year. In the case of Mantoux positive controls, tests were carried out only once.

It was found that Mantoux reaction had no correlation with LT, MIF, Myco IF and List IF. Both MIF and Myco IF, were significantly elevated in improving patients, whereas increase in List IF was not significant. An important finding was that Myco IF was at a higher level in improving patients whereas in those not responding to chemotherapy it was low.

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The recent increase in research into the immunology and the host-parasite relationship in tuberculosis can be ascribed to a number of factors, including *M. intracellulare* infection in acquired immune deficiency syndrome (AIDS) patients and the failure of bacille Calmette-Guérin (BCG) trials in South India [1]. Interest has also been focused in this area because the disease has not been effectively controlled in developing countries in spite of the availability of efficacious drugs for tuberculosis treatment [2].

The host response to stimulation with mycobacterial antigens is varied because of their complex antigenic structure. The specifically sensitized T-lymphocytes release immunological mediators in the presence of the antigen which subsequently sequester and cause activation of the macrophages (Mφ). Two of the several factors mediated by the T-lymphocytes are migration inhibitory factor (MIF) and mycobacterial inhibitory factor (Myco IF) or growth inhibitory factor (GIF) [3]. KLUN and YOUMANS [4] found Myco IF to be specific and chemically different from nonspecific MIF. They suggested that the factor could be responsible for protective immunity in tuberculosis. TURCOTTE et al. [5] corroborated this finding.

In earlier work, PERUMAL [6] applied the technique of Youmans to evaluate the role of these two factors, i.e. MIF and Myco IF, in resistance to tuberculosis in humans, using guinea-pig peritoneal Mφ. The present work represents a more extensive continuation of this earlier work, wherein the role of these two factors, MIF, which is a correlate of delayed hypersensitivity (DH), and Myco IF, which is a correlate of protective immunity, is evaluated in two classes of patients, those responding and those not responding to chemotherapy. Additionally, detailed studies and comparisons of effects of factors such as skin sensitivity (Mantoux), lymphocyte transformation (LT) after stimulation with phytohaemagglutinin (PHA) and purified protein derivative (PPD), and list inhibitory factor (List IF) are also reported.

**Materials and methods**

**Subjects**

Fifty one freshly diagnosed, sputum positive, untreated pulmonary tuberculosis (TB) patients of both sexes aged 20–40 yrs were included in this study. They were chosen randomly from drug trials of two regimens conducted at the New Delhi TB Centre. Twenty five Mantoux negative, BCG vaccinated individuals were chosen for comparison. Twenty five non-tuberculosis Mantoux positive individuals, worklings in the New Delhi TB Centre, and whose X-rays were normal, were also included in the study as controls.
As the patients were chosen at random from short-term chemotherapy trials, guidelines for human experimentation prevailing in this country were followed. The controls were volunteer workers and family contacts.

Clinical assessment

TB patients were examined clinically, for previous illness, blood, stool and routine urine examination, haemoglobin, total red blood count (RBC), total and differential white blood cell (WBC) count alanine amino transferase (ALAT) and aspartate amino transferase (ASAT). The tests were carried out at the initiation of the study and at intervals of three months after the start of treatment, and were repeated at the completion of the treatment. In case of hepatotoxicity, liver function tests, i.e. bilirubin and thymol turbidity, were carried out immediately. A chest X-ray was taken along with these tests. If deterioration of condition was suspected at any stage, X-ray was repeated even if not due. In making radiological assessment, the code recommended by the Tuberculosis Association of India was followed. Mantoux positive individuals were examined only once at the beginning of the treatment.

Treatment

Patients were chosen from two drug regimens, one of which included rifampicin whilst the other did not. These regimens were used to assess patient compliance and acceptability. The two regimens administered were as follows (figures are mg of drug administered per kg patient weight per day for 28 weeks):
1) (non-rifampicin, administered to 24 patients): streptomycin (SM), 750; Isoniazid (INH), 300; Pyrazinamide (PZ), 750; Ethambutol (ETH), 20;
2) (rifampicin - inclusive, administered to 27 patients): SM, 750; INH, 300; ETH, 20; Rifampicin (R), 600.

Bacteriological investigations

Two specimens of sputum were collected from the patients at each instance, one being spot collection and the other an overnight collection. If the patient had no sputum, cough was induced by tickling the throat; the material spat out was examined in place of sputum. All specimens were examined by fluorescent microscopy for direct smear examination and were cultured on Lowenstein Jensen (LJ) medium using Nassau's method with oxalic acid [7].

Immunological investigations

These investigations were carried out in TB patients at the initiation of the treatment and subsequently every three months up to 12 months. In the control group, investigations were carried out only once, at the start of the study. In BCG subjects, the tests were also repeated after 3 weeks. The tests were carried out by the same technician, who was trained for the particular test, throughout the period of the investigation. The technician did not know the identity of the patients.

Skin tests. One tuberculin unit TU of PPD RT 23 (BCG Vaccine Laboratory, Guindy, Madras, India) was used. Induration of more than 10 mm diameter was marked as positive. Subjects with reactions of <10 m were tested with 10 TU of PPD immediately, in order to eliminate the booster effect of the first test. This booster effect is a possibility in cases of persons exposed to mycobacterial antigens several years before but presently showing an insignificant reaction.

Separation of lymphocytes. Lymphocytes were separated from 30 ml of heparinized venous blood with Lymphoprep (Nyegaard & Co., Oslo, Norway). Total count and viability with trypan blue were estimated.

Lymphocytes transformation (LT). The macroculture method of Jusèe et al. [8] with phytohaemagglutinin (PHA) and purified protein derivative (PPD) was used to determine the immune competence and specific lymphocyte transformation against tuberculosis. The radioactivity was measured in a liquid scintillation counter (Beckman LS 3133) and LT was expressed in terms of a stimulation index (SI) as follows:

\[ SI = \frac{\text{Disintegrations per min (dpm) of culture stimulated with antigen}}{\text{Disintegrations per min (dpm) of unstimulated culture}} \]

Collection of peritoneal exudate cells (PEC). Pooled guinea-pig PEC induced by light mineral oil were collected in Eagle's minimum essential medium (MEM) with 15% heat inactivated guinea-pig serum and antibiotics.

Separation of lymphocyte culture supernatant fluid (lymphokines). Culture supernatant of lymphocyte stimulated with M. tuberculosis (H37Ra) were used as lymphokines for MIF, Myco IF and List IF (lymphokines).

Assay of migration inhibitory factor (MIF). This was carried out according to the method of Rocklin [9]. Area of migration was measured on a graph paper with a camera lucida. The percentage of migration inhibition was calculated according to the following formula:

\[ \text{Percentage of migration inhibition} = \frac{100 - \text{area of migration with lymphocyte supernatant}}{\text{area of migration without lymphocyte supernatant}} \times 100 \]

Mycobacterial growth inhibitory factor (Mycob IF). The assay of this factor was carried out by the method of CAHALL and YOUIMANS [10] with modifications using
guinea-pig PEC. Lymphocytes were separated from patients’ blood. MEM was replaced by Rosewell Park Memorial Institute medium 1640 with L-glutamate (RPMI). The optical density (OD) of the M. tuberculosis H37Rv (H37Rv) culture suspension was adjusted to 0.2–0.3, at 540 nm with a Beckman spectrophotometer (Spectronix 21). This usually gives a viable count of 10,000–15,000 bacilli/ml.1 After infection with H37Rv the Leighton’s tubes were incubated at 37°C in humid chamber with 5% CO₂. After 1 h three coverslips were fixed with neutral buffered formalin for 15 min. These served as control for the initial count of intracellular bacilli.

The lymphokine was diluted with medium containing 55% MEM, foetal calf serum (FCS), 100 U of penicillin·ml⁻¹ and 2 μg of Fungizone·ml⁻¹. Twelve tubes, with supernatant without antigen served as controls. The macrophage cultures were incubated at 37°C. Three tubes were removed after 24 h, washed in physiological saline and fixed in formalin. The medium was changed in the remaining tubes whenever necessary. After the third and sixth day three coverslips were removed and fixed in formalin and stained with Ziehl Neelson stain.

To estimate the extent of inhibition, two hundred infected Ms were examined on each of the three coverslips. The Ms were divided into two groups: those containing >10 and those containing <10 bacilli·cell⁻¹. To provide an indicator of the mycobacterial growth inhibitory effect of supernatant fluids, the percentage increase in macrophages with <10 bacilli in culture without the supernatant was taken as an indicator of inhibition and was calculated by the following formula:

\[
\text{Percentage increase} = \frac{\text{No. of Ms with <10 bacilli in test}}{\text{No. of Ms with <10 bacilli in control}} \times 100
\]

The increase in percentage of macrophages containing <10 bacilli was directly related to the degree of inhibition of the intracellular growth of the virulent mycobacteria.

Listerial growth inhibitory factor (List IF). The assay of this factor was based upon the method of Cole [11] and employed the virulent strain of Listeria monocytogenes serotype LA. The method was substantially similar to the one used for M. tuberculosis except that cultures were incubated for 12, 24, 48 and 72 h. All coverslips were removed and cells counted at 72 h. Listeria per macrophages was calculated by the following:

\[
\text{Percentage decrease} = \frac{100 \times \text{Listeria per macrophage in test}}{\text{Listeria per macrophage in control}} \times 100
\]

The percentage decrease was directly related to the degree of inhibition of the intracellular growth of Listeria monocytogenes.

Statistical analysis

Patients were selected randomly from short term chemotherapy trials conducted at the New Delhi TB Centre, New Delhi.

In order to establish the uniformity of the population within the treatment group, variance analysis with F statistics were carried out with immunological parameters in TB patients and BCG vaccinated individuals. To establish the association between responding and non-responding treated patients, Pearson product moment coefficient and linear regression analysis were conducted as a function of sputum culture. Values of the regression line intercept and slope were determined in the case of MIF, Myco IF and List IF.

The significance of the difference between the means of the various immunological parameters at successive stages of treatment was tests by Student’s t-test.

Clinical assessment

Radiological. The radiological assessment of these patients at the beginning of treatment showed that out of 51 cases, 24 had infiltration, 13 had cavity, and 14 had infiltration with cavity. Sixteen of the patients had relapse and showed no improvement. Thirty five patients showed distinct improvement. The patients were treated for a period of 28 weeks and the condition of the patients at a period of 12 months (i.e. six months after treatment was completed) was considered for final assessment of prognosis.

Bacteriological. Cultural assessment showed a total of nine relapses, seven in group A and two in group B. Three out of seven were resistant to INH and one to INH and EMB. The remaining three were sensitive to all of the drugs. One of the two relapses belonging to treatment group B was resistant to INH whereas the other was sensitive to all drugs. As bacteriological assessment is more specific than radiological assessment this criterion was taken for analysis. However, the above nine cases also showed radiological deterioration and so they were taken as relapse cases.
### Immunological assessment

Table 1 gives changes in immunological parameters of the individuals at various stages. The variance analysis of the immunological data showed that TB patients were drawn from a single population, as values of F were less than 1.0 in all of the tests. However, in BCG vaccinated individuals, F values were slightly high, ranging between 2.0–4.0.

**Skin test.** Whereas the Mantoux reaction in BCG vaccinated individuals increased after three months and reached that of TB patients at the end of nine months, the reaction for TB patients did not show significant change during the test period. The reaction of controls was similar to those of TB patients. Although in the case of patients responding to chemotherapy the Mantoux response was at a higher level as compared to those not responding to chemotherapy, the difference was not significant.

#### Table 1. Changes in skin test reaction, LT, MIF, Myco IF, and List IF during the course of treatment in TB patients, Mantoux negative BCG vaccinated individuals and non-TB patients

<table>
<thead>
<tr>
<th>Period</th>
<th>Skin test</th>
<th>PPD</th>
<th>LT</th>
<th>PHA</th>
<th>MIF</th>
<th>Myco IF</th>
<th>List IF</th>
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<tbody>
<tr>
<td>Months</td>
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<tr>
<td>TB patients (n=51)</td>
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<tr>
<td>0</td>
<td>13.0±2.71</td>
<td>4.4±1.45</td>
<td>35.6±17.36</td>
<td>63.4±8.78</td>
<td>57.4±16.85</td>
<td>37.7±18.21</td>
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<td>3</td>
<td>13.6±3.29</td>
<td>4.8±1.45</td>
<td>52.2±24.85</td>
<td>66.6±13.28</td>
<td>62.2±18.21</td>
<td>36.9±15.71</td>
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<td>6</td>
<td>15.1±3.86</td>
<td>5.1±1.64</td>
<td>62.5±28.12</td>
<td>61.6±28.7</td>
<td>62.3±19.00</td>
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<td>17.4±2.64</td>
<td>5.8±2.07</td>
<td>73.3±38.64</td>
<td>61.9±15.93</td>
<td>62.5±22.64</td>
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<td>12</td>
<td>18.7±5.43</td>
<td>5.7±3.64</td>
<td>75.4±35.78</td>
<td>61.5±15.28</td>
<td>67.3±21.21</td>
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<td>Mantoux negative BCG vaccinated individuals (n=25)</td>
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<td>0</td>
<td>Negative</td>
<td>0.3±0.2</td>
<td>5.5±3.9</td>
<td>16.0±5.3</td>
<td>7.1±3.4</td>
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<tr>
<td>3rd week</td>
<td>Negative</td>
<td>0.8±0.2</td>
<td>8.6±3.9</td>
<td>35.8±5.6</td>
<td>18.6±8.0</td>
<td>11.8±5.3</td>
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<td>3</td>
<td>12.5±3.4</td>
<td>1.7±0.9</td>
<td>22.6±14.3</td>
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<td>9</td>
<td>18.5±5.1</td>
<td>2.6±1.3</td>
<td>32.6±15.6</td>
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<tr>
<td>12</td>
<td>20.2±5.8</td>
<td>2.7±1.6</td>
<td>35.2±15.4</td>
<td>58.5±10.1</td>
<td>43.5±14.2</td>
<td>32.2±12.2</td>
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<td>Non-TB controls (n=25)</td>
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<tr>
<td>0</td>
<td>14.7±3.3</td>
<td>4.8±2.10</td>
<td>42.1±15.4</td>
<td>69.8±5.9</td>
<td>50.4±16.1</td>
<td>39.4±21.8</td>
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</tbody>
</table>

Data are presented as mean±sd. LT: lymphocyte transformation; MIF: macrophage migration inhibitory factor; Myco IF: myobacterial growth inhibitory factor; List IF: listeria growth inhibitory factor; TB: tuberculosis; BCG: bacille Calmette-Guerin; PPD: purified protein derivative; PHA: phytohaemagglutinin.

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**Fig. 1.** Tuberculin skin sensitivity Mantoux (A) Lymphocyte transformation with PPD (B) and PHA (C) in pulmonary tuberculosis patients during chemotherapy. Comparison of responding and non-responding individuals as assessed by sputum culture. The differences between values of responding and non-responding individuals were not significant. ▲ ▲: Sputum culture negative; ▲ — ▲: Sputum culture positive; PPD: purified protein derivative; PHA: phytohaemagglutinin.
Lymphocytes transformation (LT). To estimate the immune competence of subjects, LT stimulation with PHA (LT/PHA) was determined. LT stimulation with PPD (LT/PPD) was used to indicate specific sensitization. The initial values for both PHA and PPD stimulation were much lower in BCG vaccinated individuals and never reached those of TB patients or controls.

LT/PPD did not show significant increase in TB patients over a period of 12 months. LT/PHA reached very high mean values for TB patients, which indicated improvement in their immunocompetence.

There was no significant difference at any stage in the values of LT/PPD in patients responding to chemotherapy and those not responding to chemotherapy. In patients who improved in response to chemotherapy, the values of LT/PHA were higher than in patients who did not improve. Thus, it can be seen that immunocompetence of responding patients was higher than non-responding individuals, although the difference did not always attain statistical significant (fig. 1). Pearson product moment correlation coefficient for estimation of regression showed that in the case of BCG vaccinated individuals there was a very weak relationship between skin test, LT/PPD and LT/PHA. On the other hand, TB patients had a correlation coefficient nearing 1.0 (0.997 or 0.999), thus indicating a strong correlation between the three tests.

MIF, Myco IF and List IF. MIF values did not show any increase over the initial value during the 12 month in TB patients. However, in BCG vaccinated individuals the increase was substantial. Both Myco IF and List IF increased over the test period in TB individuals. It is interesting to note that BCG vaccinated Mantoux negative individuals showed significant increase in all parameters of CMI. There was no significant difference in the values of MIF, Myco IF and List IF in patients with the two treatment regimens.

The differences in immunological parameters as related to prognosis of disease were observed. The mean values of MIF, Myco IF, and List IF in improved patients (sputum culture negative) were significantly higher than those in patients who did not show improvement (culture positive). There was no significant difference in List IF between the two groups at 3 months. However, at 6, 9 and 12 months the difference became highly significant (p<0.001) in individuals responding to chemotherapy (fig. 2).

Linear regression analysis and correlation coefficient estimation were carried out to correlate sputum culture and MIF, Myco IF and List IF tests. The r values for MIF were 0.87 and 0.5 for culture positive and culture negative patients, respectively. However, in the case of Myco IF and List IF they were 0.815 and 0.79 for culture positive patients but 0.9997 and 0.99 for culture negative patients, thus indicating a strong relationship between prognosis and the test especially in the case of culture negative individuals.

**Fig. 2.** Changes in mean values of MIF, Myco IF and List IF in pulmonary tuberculosis patients during chemotherapy. Comparison of responding and non-responding individuals as assessed by sputum culture. MIF is expressed in percentage inhibition of migration, Myco IF in percentage increase in number of macrophage with <10 bacilli and List IF in percentage decrease in L. monocytogenes per macrophage. *: p<0.01 differences between responding and non-responding patients; \(\square\): sputum culture negative; \(\square\): sputum culture positive; MIF: macrophage migration inhibitory factor; Myco IF: mycobacteria growth inhibitory factor; List IF: listeria growth inhibitory factor.
Discussion

Our results show that Myco IF values were higher in individuals responding to chemotherapy and lower in non-responding patients. On the other hand the values of MIF levels remained more or less constant from 3 months onwards, although they were at higher levels in responding patients. The present study shows that both DH and cell-mediated acquired resistance play a role in anti-bacterial mechanisms in tuberculosis, as evidenced by the reported levels of Myco IF and MIF in TB patients.

DANNENBERG [12] postulated that products elicited at lower levels by DH, such as MIF or other lymphokines, keep the inflammatory processes active, making the site of lesion unsuitable for the growth of bacteria. He suggested that DH can be beneficial to the host when it develops slowly and involves inflammatory reactions that help to localize the bacteria, whereas Myco IF plays a role as a specific antibacterial factor.

Our studies show that the difference in immunocompetence of an individual is related to his response to PHA. The cells were equally activated by PPD in both groups of patients (responding to treatment and non-responding). The immune competence of the patients responding to chemotherapy however was more than that of patients not responding to treatment as seen by their response to PHA (fig. 1). It may be argued that the stimulation or otherwise of the immune competent T-cells by Myco IF determines the anti-tuberculous immunity and response to drug treatment.

We feel that the present study may be significant in its contribution to the understanding of the mechanics of specific immunity. To our knowledge this is the first time that the role played by two separate factors of the sensitized lymphocytes in activation of Mac has been demonstrated in tuberculous patients. The specific nature of Myco IF is manifested by the fact that the increase in anti-listerial response was shown in the study to be less significant than the anti-mycobacterial response. MIF as a parameter of DH was also shown to be elevated in improving patients. Cox et al. [13] have recently found association between HLA D3 and HLA DR antigens and immune responsiveness of Mexican American TB patients, although this was not related to the disease. Our studies on the other hand appear to indicate that the production of Myco IF by T-lymphocytes perhaps determines the outcome of the disease. The genetic markers on the lymphocytes may regulate production of Myco IF which in turn regulates the response of patients to tubercle bacilli and chemotherapy.

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References


Association entre un facteur inhibiteur de la croissance mycobactérienne et l'immunité anti-tuberculeuse. S. Chandrasekhar, V.K. Perumal. RÉSUMÉ: Les mécanismes d'immunité à médiation cellulaire (CM) ont été étudiés chez 51 patients atteints de tuberculose pulmonaire pour évaluer le rôle du facteur d'inhibition de la croissance mycobactérienne dans le pronostic de l'infection, avant et après administration des médicaments anti-tuberculeux. Vingt-cinq individus Mantoux négatif, qui avaient été ultérieurement vaccinés au BCG, et vingt-cinq sujet Mantoux positif, non tuberculeux, pris comme contrôles, ont été inclus dans l'étude. Leur étude clinique a été comparée avec la sensibilité cutanée (test de Mantoux); la transformation lymphocytaire (LT) après stimulation par phythômégagglutinine (PHA) et par la PPD; le facteur d'inhibition de la migration des macrophages (MIF), le facteur inhibiteur de la croissance mycobactérienne (Myco IF) et le facteur inhibiteur de la croissance des Listeria (List IF). Ces tests ont été exécutés au début du traitement et à des intervalles de trois mois, allant jusqu'à un an. Dans le cas des contrôles Mantoux positif, les tests n'ont été exécutés qu'une seule fois.

L'on a trouvé que la réaction de Mantoux n'avait pas de corrélation avec LT, MIF, Myco IF et List IF. MIF et Myco IF, tous deux, s'avéraient significativement élevés chez les patients en amélioration, alors que l'augmentation de List IF est non significative. Une observation importante est que Myco IF est à un niveau plus élevé chez les patients en amélioration, alors qu'il est bas chez ceux qui ne répondent pas à la chimiothérapie. *Eur Respir J*, 1991, 4, 783–788.