CMI Response of Tuberculosis Patients and Volunteers to Mitogens and Mycobacterial Antigens by LTT.

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Introduction

Various mechanisms have been proposed in the past to explain the inability of the body's cell mediated immunity (CMI) to cope with the infecting organism in myco bacterial disease such as leprosy. They include short lived suppressor cells, Interleukin-2 (IL2) defect and Prostaglandin mediated suppression[1,2,3]. In leprosy these mechanisms have been studied using the lymphocyte transformation test (LTT) to elucidate CMI *in vitro*.

The present study was designed to study the regulation of CMI in tuberculosis patients and normal individuals with regard to induction, expression, inhibition and modulation due to prior exposure to environmental mycobacteria.

Materials and Methods

Patients: All patients included in this study were previously untreated smear and culture positive cases attending out-patient clinic at Tuberculosis Research Centre (TRC), Madras.

Controls : Volunteers from the staff members of the TRC were included as normal healthy controls.

Lymphocyte transformation test : From tuberculosis patients and normal subjects, 20 ml. of peripheral blood was drawn and the lymphocytes were isolated by

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Ficoll Hypaque density gradient centrifugation. Ficoll Hypaque or Lymphocyte separation medium (LSM, Bionetics, Kensington, Maryland 20795, U.S.A.) was overlayered with peripheral blood, in a ratio of 1 : 1, and centrifuged at 1508 rpm for 30 minutes. Lymphocytes were removed from the interphase, washed thrice with Hank's balanced salt solution and adjusted to a density of 0.5 x 10^6 cells/ml in RPMI-1640 (Grand Island Biological Company, New York, U.S.A.) after assessing the viability of the lymphocytes using the Trypan Blue dye exclusion method. The viability of the cells was around 95%.

Cells were cultured in 0.2 ml of RPMI-1640 supplemented with Penicillin (100 IU/ml of medium), Streptomycin (100 µg/ml), Glutamine (300 µg/ml) and 10% autologous plasma in 96-well U-bottomed tissue culture plates. (Dynatech Laboratories, Inc., Alexandria, Virginia, U.S.A.)

The cultures were stimulated with mitogens Phytohaemagglutinin (PHA) 5 µg/ml (Wellcome Reagents Ltd., Bakenham, England), Phorbal Myristic Acid (PMA) 600 µg/ml and Conconavalin-A 50 µg/ml (Con-A) (Sigma Chemical Co., St. Louis, MO, U.S.A.).

The antigens used were *M. tuberculosis* $H_{37}Rv$, *M. tuberculosis* 7219-South Indian strain, British strain of *M. tuberculosis* S1, *M. bovis, M. scrofulaceum, M. kansasii, M. fortuitum, M. terrae, M. avium intracellulare* serotype 8 (S8), *M. chelonei, M. flavescens, M. bovis* (BCG) and purified protein derivative of *M. tuberculosis* (PPD) (gift from the Ministry of Agriculture, Fisheries & Food, Weybridge, England). All mycobacterial antigens were used at a concentration of 10 ug/ml. The preparation of these antigens is as described in the previous paper. Optimal concentrations of the antigens as well as optimal length of incubation period of the culture were fixed from prior standardisation experiments with normal subjects. Whenever the batch of the mitogens or antigens was changed, the dosages were checked.

Mitogen-stimulated cultures as well as antigen-stimulated cultures were incubated at 37°C in an atmosphere of 5% CO_2 for 144 hrs. The proliferative response was measured after adding 1.0 uCi of 3H–Thymidine (Specific activity 13,000 uCi/mm) (Bhabha Atomic Research Centre, Bombay) and incubating for a further period of 18 hours at 37°C before harvesting.

Cells were harvested with multiple automatic specimen harvester-Mash-II (Microbiological Associates, U. S. A.) and deposited on glass tissue filter paper (Whatman Inc., Litton, NJ., U.S.A.). These paper discs were transferred to biovials (Beckman instruments Inc., California, U.S.A.), with 2.5 ml of scintillation fluid containing 4.0 g/litre of PPO and 0.05 g/litre of POPOP (Sigma Chemical Company,

St. Louis, MO, U.S.A.) in toluene and counted for 30 seconds in Packard Tricarb-300 (Packard Instruments Inc., III., U.S.A.).

Removal of adherent cells : Total mononuclear cells from the patients and the normal volunteers were seeded in plastic petri dishes. The cells were supplemented with 10% heat inactivated (for 30 minutes at 56°C) pooled normal human serum and incubated at 37°C for 90 minutes in 5% CO_2 atmosphere. The supernatant containing the non-adherent cells was removed. No effort was made to wash and take out the loosely bound cells. The cell density of the non-adherent cells was adjusted to be 0.5 x 10^6 cells/ml.

Short lived suppressor cells *in-vitro :* Mononuclear cells from the patients and the volunteers were cultured in the absence of antigen or mitogen for 24 hours or 48 hours. After 24 or 48 hours, the antigens and mitogens were added to the culture and LTT was done as described above.

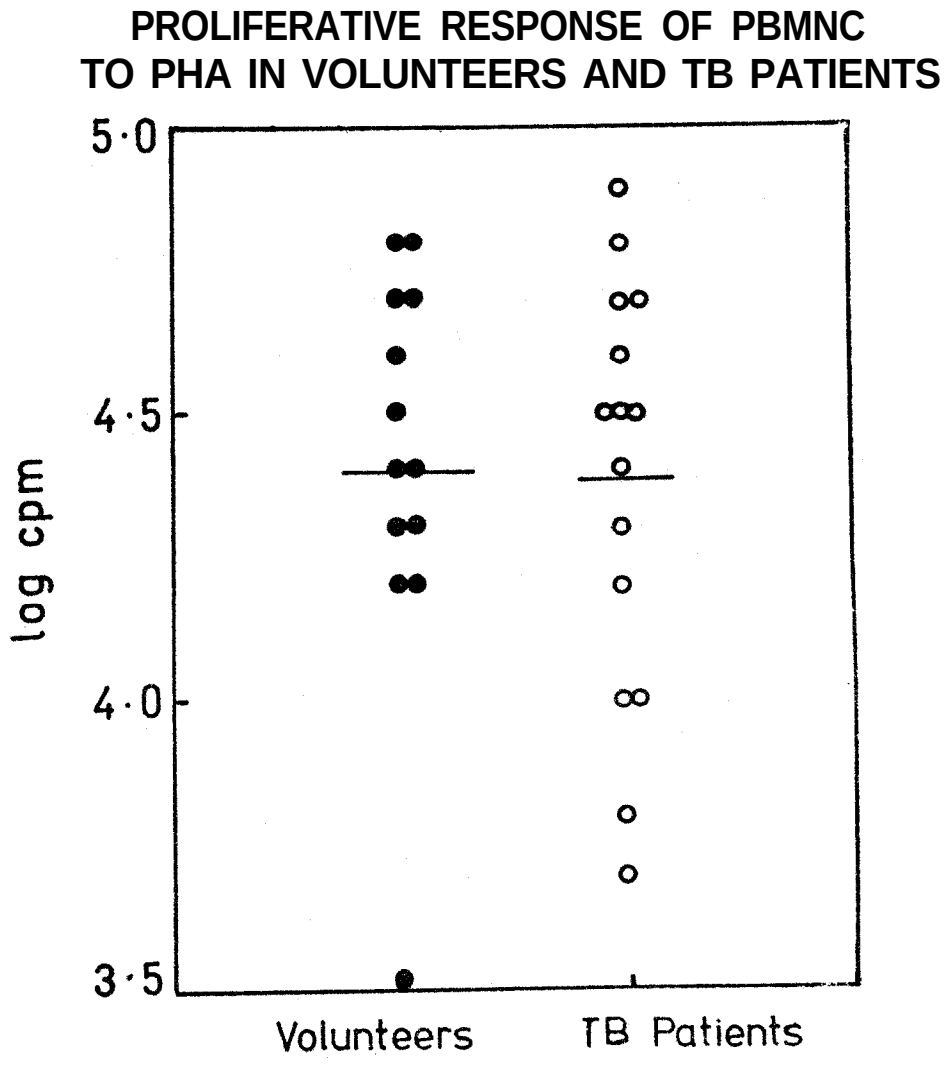
Co-stimulation with antigen : LTT was done with PBMNC in the presence of *M. tuberculosis* $H_{37}Rv$ alone and $H_{37}Rv$ along with the following antigens ; *M. tuberculosis* 7219, *M. scrofuloceum, M. avium intracellulare* serotype 8, *M. bovis (BCG),* and *M.flavescens.* All these antigens were added in 10 µg/ml concentrations. Also PBMNC of tuberculosis patients and volunteers were cultured *in-vitro* in the presence of 20 µg/ml of mycobacterial antigens along with any one of the following three doses of Con-A, 5 µg/ml, 10 µg/ml and 20 µg/ml.

Experiments with indomethacin : PBMNC and non-adherent cells were separately cultured with *M. scrofulaceum, M. bovis (BCG) M. avium intracellulare* serotype 8, *M. chelonei* and *M. tuberculosis* $H_{37}Rv$ in the presence and absence of indomethacin 1 mg/ml.

Results

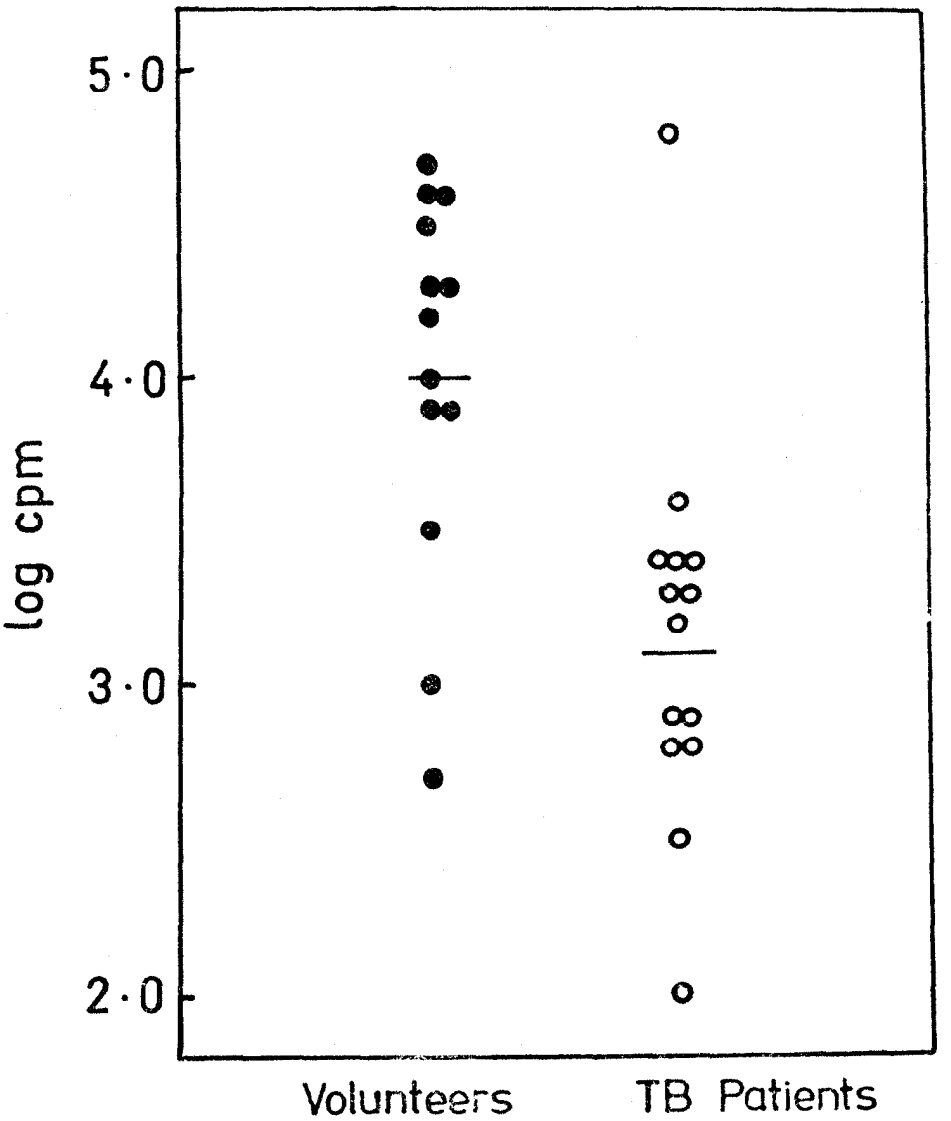
The mean log cpm ± SD of 1.5 tuberculosis patients and 12 volunteers to PHA, Con-A and PMA were 4.4 ± 0.4 ; 3.1 ± 0.6 and 4.0 ± 0.6 ; 3.4 ± 0.8 and 40 ± 0.7 respectively.

There was significantly lower blastogenic response to Con-A in tuberculosis patients than in volunteers. Between the two groups no significant difference was seen in the blastogenic response to PHA and PMA (Figs. 1, 2 and 3).

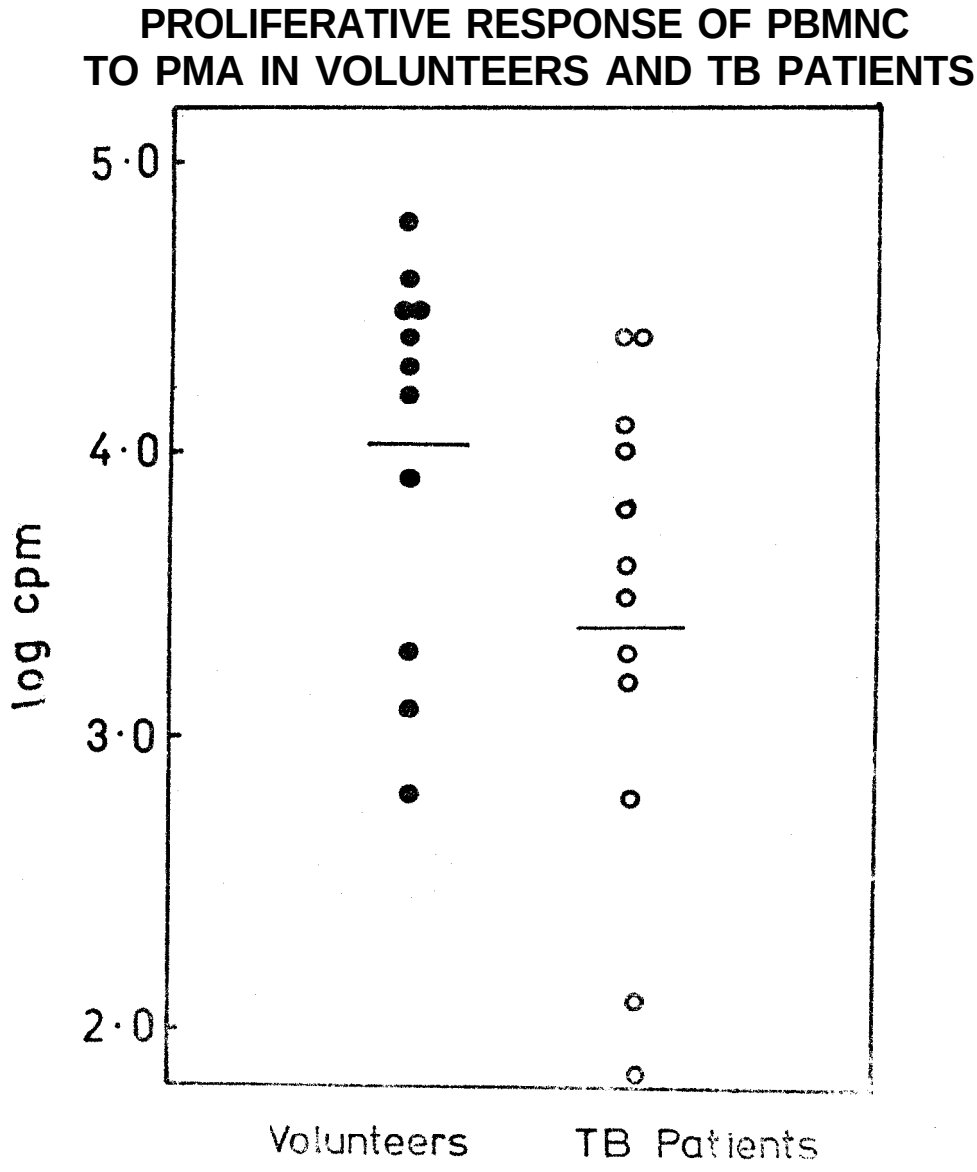

PROLIFERATIVE RESPONSE OF PBMNC
TO PHA IN VOLUNTEERS AND TB PATIENTS
5·0
4·5
4·0
3·5
log cpm
Volunteers
TB Patients

Figure–1.

PROLIFERATIVE RESPONSE OF PBMNC TO CON A IN VOLUNTEERS AND TB PATIENTS

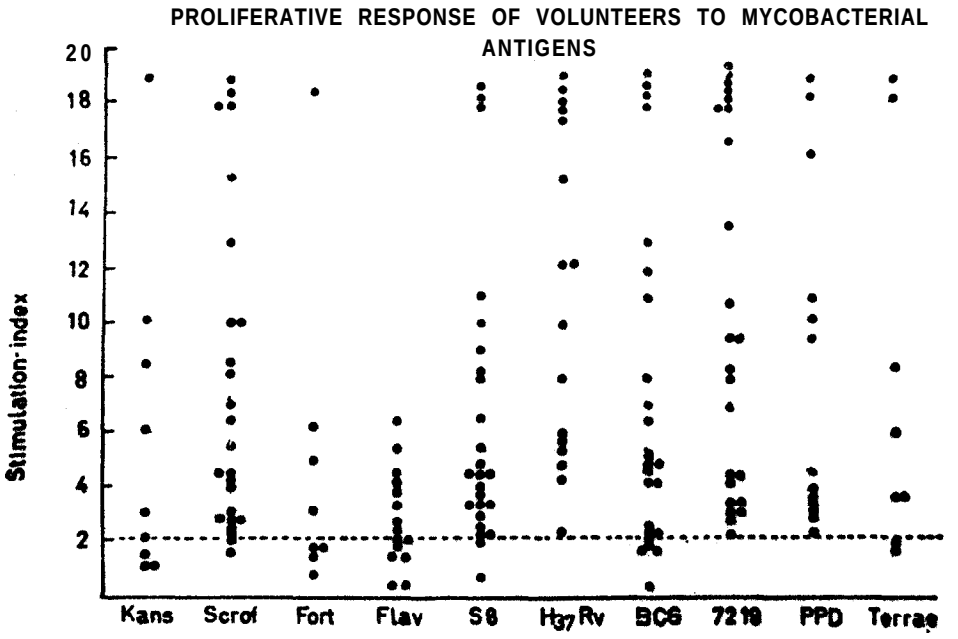


Figure–2

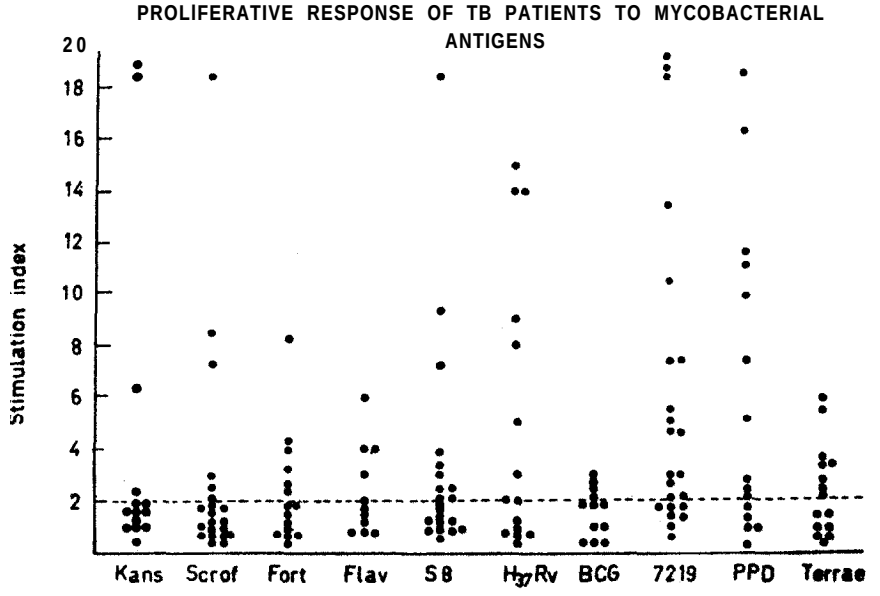


Figure—3.

The stimulation index obtained with these various antigens are shown in figures 4 and 5. Generally the response of the lymphocytes from most of the normal individuals to the antigens *M. bovis* (*BCG*), *M. kansasii*, *M. avium intracellulare* serotype 8, *M. scrofulaceum*, *M. terrae*, *M. fortuitum* and *M. chelonei* were lower, with a few showing suppression, that is a stimulation index of less than 2, when compared to the response to *M. tuberculosis* 7219, *M. tuberculosis* $H_{37}Rv$, *M. tuberculosis* S1 and PPD.



Figure–4



Figure–-5

Among patients, a stimulation index of less than 2.0 was seen in 7 out of 23 patients to *M. tuberculosis* 7219, PPD and *M. terrae,* 15 out of 22 patients to *M. scrofulaceum,* 10 out of 15 patients to *M. kansasii,* 12 out of 22 patients to *M. avium intracellulare* serotype 8, 8 out of 21 patients to BCG, 9 out of 15 patients to *M. flavescens* and 6 out of 15 patients to *M. tuberculosis* $H_{37}Rv$. Thus more number of patients showed suppression to mycobacterial antigens than volunteers. Hence investigations were extended to learn the nature of suppressive response of the PBMNC from tuberculosis patients to various antigens.

Short lived suppressor cells :

Various mycobacterial antigens were added to PBMNC cultures from 3 patients at 0 and 24 hours after initiation of the culture. It is seen from figure 6 that preculture for 24 hours before the addition of antigens did not cause any consistent enhancement in the response.

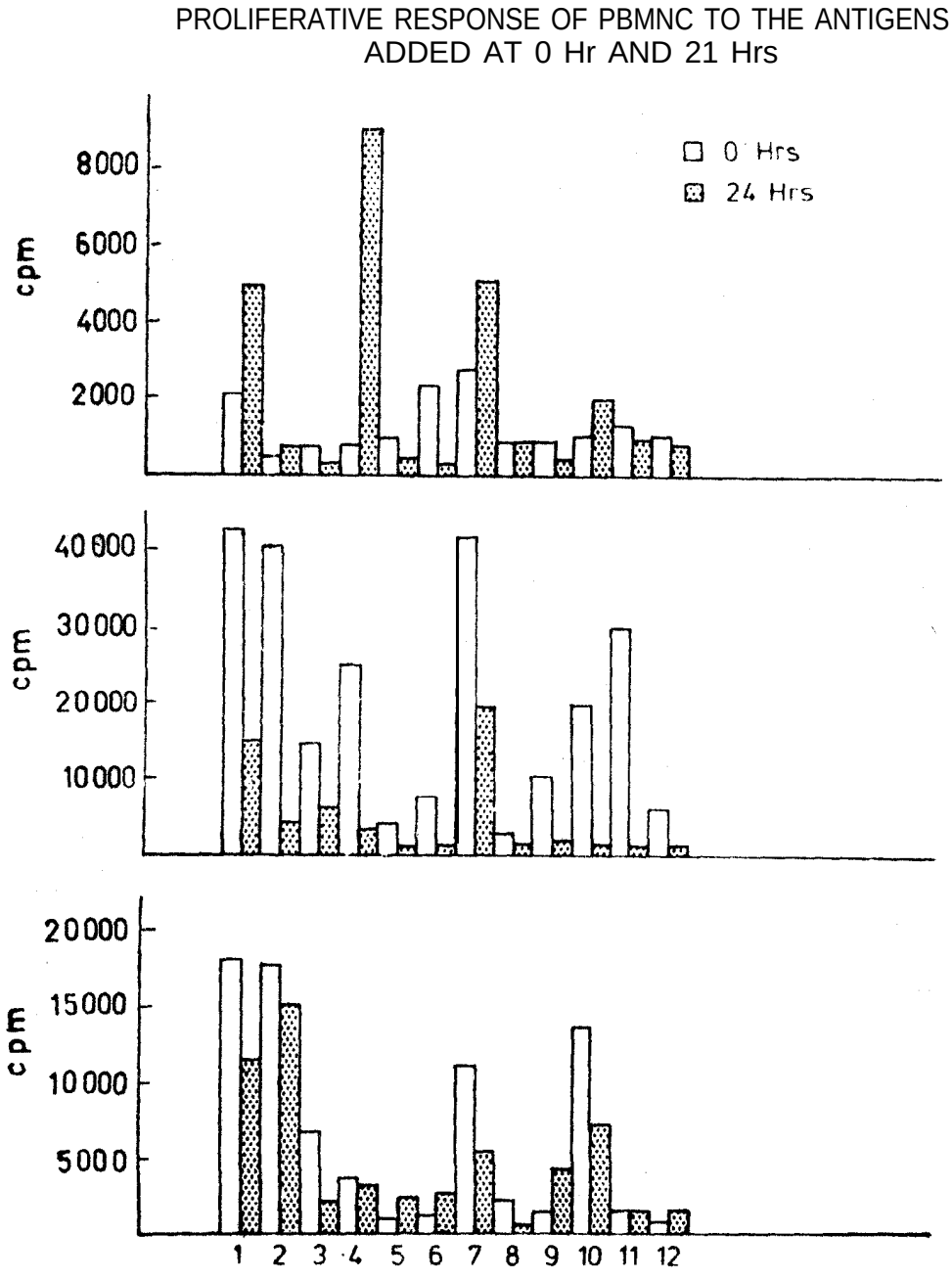
Table 1 shows the effect of delaying the addition of mitogens for 48 hours and 144 hours on the proliferative responses of PBMNC from patients and from volunteers. Out of the 6 volunteers studied, four showed enhanced response to Con-A and PMA while two showed enhanced response to PHA when they were added after 48 hours. Out of the 2 patients, addition after 48 hours enhanced the response to Con-A in both the patients and similar enhancement was observed to PHA and PMA in 1 patient. When mitogen addition was delayed to 144 hours, similar response was observed. Addition of *M. tuberculosis* antigens and other mycobacterial antigens when delayed by 48 hours produced no consistent enhancement in lymphocyte response (Tables 2 and 3).

Table – 1

The effect of delaying the addition of mitogens for 48 and 144 hrs on the proliferative responses of PBMNC from patients and from normal volunteers.

Mitogcns	48 hours		144 hours	
	Normal volunteers	Patients	Normal Volunteers	Patients
Con-A	4/6	2/2	4/10	2/9
PHA	2/6	1/2	9/10	3/9
PMA	4/6	1/2	6/10	2/9

No of individuals showing enhanced responses when mitogen addition was delayed for either 48 or 144 hrs., (over total number tested).



Figure–6

1. *M. tuberculosis*- 7219 2. *M. bovis* 3. *M. scrofulaceum* 4. *M. kansasii*
5. *M. fortuitum* 6. *M. avium intracellulare* 7. *M. tuberculosis*- S.I. *8. M. terrae*
9. *M. bovis* BCG 10. *M. chelonei* 11. *M. flavescens* 12. *M. tuberculosis* $H_{37}Rv$

Table–2

The effect of delaying the addition of homologous antigens for 48 and 144 hrs. on the proliferative responses of peripheral blood mononuclear cells from patients and from normal volunteers.

Antigens	48 Hrs.		144 Hr.	
	Normal volunteers	Patients	Normal volunteers	Patients
M. tuberculosis- 7219	5/6	2/6	2/10	3/8
M. tuberculosis- SI Strain	1/6	2/6	0/10	2/8
M. tuberculosis- $H_{37}Rv$	4/6	1/6	2 / 9	0/7
PPD-S	3/6	1/6	2/10	0/7

No. of individuals showing enhanced responses when mitogen addition was delayed for either 48 or 144 hrs. (over total number tested).

Table–3

The effect of delaying the addition of other mycobacterial antigens on the proliferative responses of PBMNC from patients and normal volunteers.

Antigens	48 Hrs.		144 Hrs.	
	Normal volunteers	Patients	Normal volunteers	Patients
M. bovis	5/6	0/2	2/9	2/8
M. scrofulaceum	3/6	1/2	0/9	3/8
M. kansasii	4/6	0/2	1/5	3/8
M. fortuitum	4/6	0/2	1/5	1/8
M. avium intracellulare	5/6	0/2	0/9	2/8
M. terrae	4/6	1/2	0/5	2/8
BCG	1/6	0/2	0/9	2/8
M. chelonei	2/6	1/2	0/5	1/3
M. flavescens	4/6	1/2	1/5	0/3

No. of individuals showing enhanced responses when mitogen addition was delayed for either 48 or 144 hrs., (over total number tested).

Effect of Addition of Indomethacin :

Out of a total of 4 volunteers and 3 patients the proliferative response of PBMNC from all 4 volunteers and 2 patients was enhanced by the addition of indomethacin. (Tables 4 and 5). This was observed with respect to various antigens like S. Indian isolate *M. tuberculosis* 7219, *M. scrofulaceum, M. avium intracellulare* serotype 8, *M. bovis* (BCG), *M. chelonei* and *M. flavescens.* However, in one of the tuberculosis patients where antigen induced proliferation was minimal, indomethacin could not induce or enhance proliferation of the cells.

Table–4

The effect of addition of indomethacin (1 μg/ml) on day '0' on the proliferative responses of PBMNC from four normal volunteers.

Soluble Antigen (20 μg/ml)	Individual tested	Mean Δ counts per minute per culture ± S.D.	
		Antigen alone	+ Indomethacin
M. tuberculosis- 7219	1	36276 ± 10718	47358 ± 16491
	2	117900 ± 2536	127736 ± 17244
	3	32463 ± 14268	53026 ± 2277
	4	99050 ± 9042	120340 ± 4601
M. scrofulaceum	1	33953 ± 14813	48896 ± 5375
	2	50416 ± 2559	82043 ± 2624
	3	28010 ± 15410	53 190 ± 1027
	4	57156 ± 11492	98826 ± 12240
M. avium intracellulare	1	19743 ± 4655	26026 ± 9381
serotype-8	2	47886 ± 3857	76076 ± 9472
	3	25196 ± 3743	28326 ± 7240
	4	55873 ± 13978	74650 ± 17154
M. bovis BCG	1	25343 ± 7117	34983 ± 2630
	2	103166 ± 7983	128535 ± 6450
	3	15565 ± 5621	53570 + 1612
	4	77916 ± 19936	119969 ± 2483
M. chelonei	1	25590 ± 5586	39536 ± 6501
	2	34520 ± 9889	43710 ± 4758
	3	37596 ± 10625	39123 ± 25525
	4	40090 ± 12682	68866 ± 20273
M. flavescens	1	5545 ± 572	10180 ± 1943
	2	30516 ± 4253	55856 ± 12991
	3	10890 ± 1626	31995 ± 1647
	4	20320 ± 5489	45430 ± 8486

Table– 5

The effect of addition of indomethacin (1 µg@ml) on day '0' on the proliferative responses of PBMNC from three tuberculosis patients.

Soluble Antigen (20 µg/ml)	Individual tested	Mean Δ counts per minute per culture ± S.D.	
		Antigen alone	+ Indomethacin
M. tuberculosis– 7219	1	1.5548 ± 4218	25944 ± 2580
	2	3631 ± 1779	4243 ± 759
	3	5441 ± 676	10417 ± 4113
M. scrofulaceum	1	4775 ± 916	9409 ± 2959
	2	1830 ± 714	2653 ± 1102
	3	4711 ± 1296	9496 ± 1797
M. avium.	1	7675 ± 3814	6577 ± 2266
intracellare	2	2835 ± 219	1768 ± 561
serotype– 8	3	6703 ± 5451	8369 ± 1467
M. bovis BCG	1	14556 ± 5575	24470 ± 2340
	2	4672 ± 521	10112 ± 14414
	3	7790 ± 1687	17994 ± 4516
M. chelonei	1	7675 ± 3814	7831 ± 3382
	2	2439 ± 40	2822 ± 202
	3	2743 ± 305	6377 ± 1902
M. flavescens	1	2876 ± 1543	2979 ± 1054
	2	2342 ± 1181	3624 ± 664
	3	3611 ± 1378	6933 ± 1460

Effect of indomethacin addition on non-adherent cells :

In 3 patients, from the total PBMNC the adherent cells were removed by plastic adherence. The resulting non adherent cells and total PBMNC were cultured in the presence of antigens and indomethacin. Indomethacin failed to cause enhancement of responses of non-adherent cells (Table–6). The results are expressed in absolute counts.

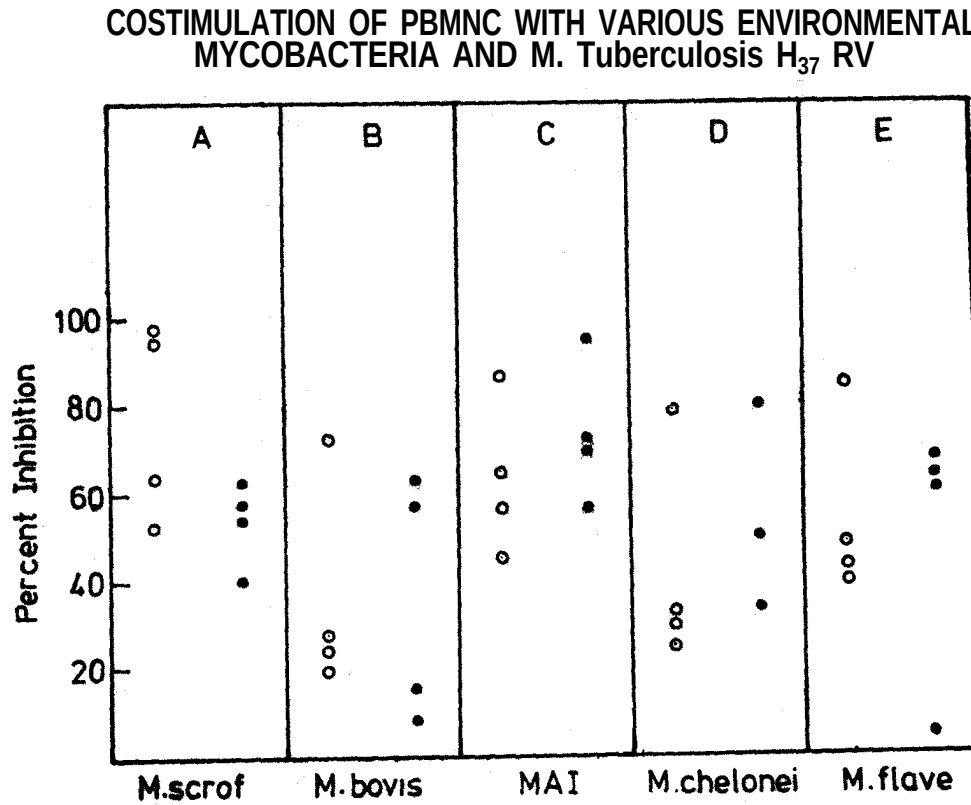
Table– 6

The effect of addition of indomethacin (1 µg/ml) on day '0' on the proliferative responses of total PBMNC and non-adherent cells from 3 tuberculosis patients.

Soluble Antigen	Individual tested	Total cells		Non-adherent cells	
		Ag. alone	+ Indo	Ag. alone	+ Indo
M. tuberculosis	1	15548	25944	2454	2249
	2	3631	4243	8847	6308
	3	5441	10217	4485	4474
M. scrofulaceum	1	4775	9409	2646	3855
	2	1830	2653	2657	3470
	3	4711	9496	3703	3178
M. avium-intracellulare serotype-8	1	7675	6577	2262	2048
	2	2835	1768	3259	2073
	3	6703	8369	–	
M. bovis BCG	1	14556	24470		
	2	4672	10112	6115	5410
	3	7790	17994	5682	4462
M. chelonei	1	7675	7871	2278	1898
	2	2439	2822	2444	2328
	3	2743	2743	2987	2387
M. flavescens	1	2876	2979	2783	2236
	2	2342	3624	2237	2340
	3	3611	6933	1933	1992

Co-stimulation of PBMNC with various mycobacterial antigens with *M. tuberculosis* $H_{37}Rv$:

The results of co-stimulation experiments with mycobaterial antigen are expressed as percent inhibition in figure 7. It was seen that BCG and *M. chelonei* induced less suppression when compared to *M. scrofulaceum, M. avium intracellulare* serotype 8 and *M,. flavescens.*



Figure– 7

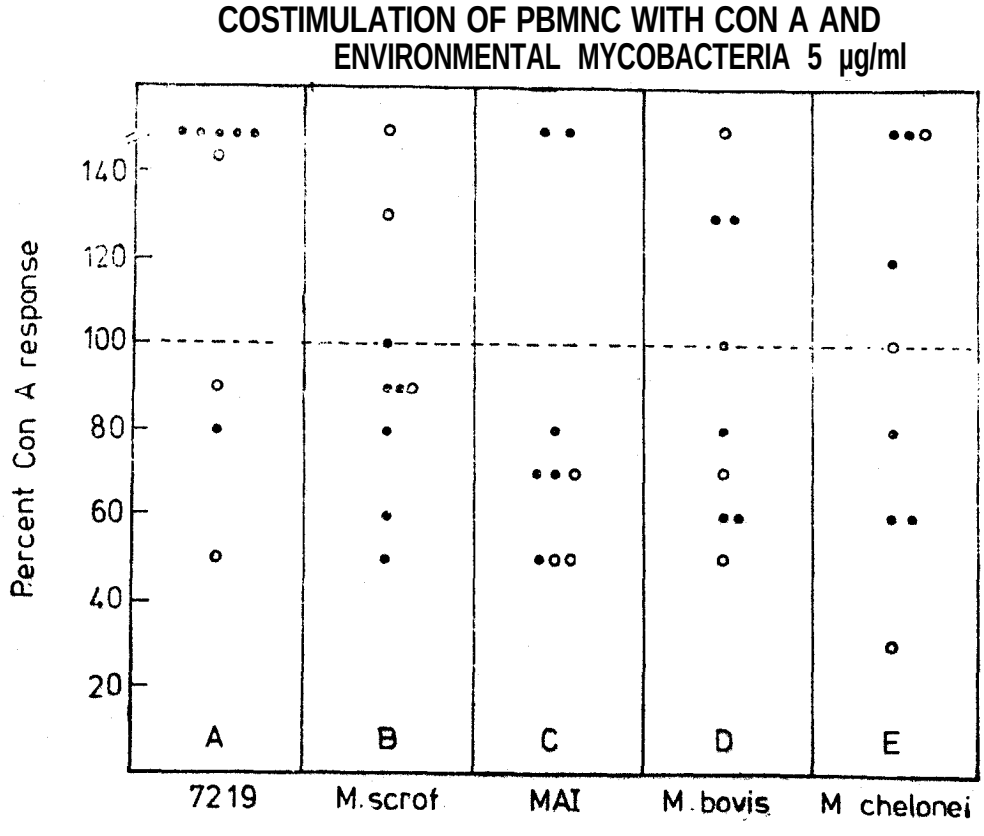
Inhibition of the *M. tuberculosis* $H_{37}Rv$ response by (A) *M. scrofulaeum,* (B) *M. bovis* BCG, (C) *M. avium intracellulare,* (D) *M. chelonei,* (E) *M. flavescens,* using PBMNC from normal volunteers (•) and pulmonary tuberculosis patients (o).

$$\text{Percentage inhibition} = \frac{100 - \text{net cpm of A\&B}}{\text{Net cpm A + Net cpm B}} \times 100$$

cpm AB = Net response obtained when *M. tuberculosis* $H_{37}Rv$ and another antigen added together.

cpm A & cpm B = Net responses obtained in cultures stimulated respectively by *M. tuberculosis* $H_{37}Rv$ and the second antigen.

Figure 8 represents percent response of PBMNC to 20 µg/ml of Con-A and Con-A along with mycobacterial antigens. The response of patients and volunteers to Con-A were not uniformly inhibited by the addition of mycobacterial antigens and enhancement was seen in a few volunteers and patients. This was observed with all the three doses of Con-A. (5 µg/ml, 10 µg/ml and 20 µg/ml).



Figure– 8

Percentage of Con-A response in lymphocytes stimulated with (A) *M. tuberculosis*- 7219 (B) *M. scrofulaceum* (C) *M. avium intracellulare* (D) *M. bovis* BCG and (E) Con-A (20 µg) for 6 days from normal volunteers (o) and patients with pulmonary tuberculosis (•).

Discussion

Though the effector functions of the proliferating cells and their relationship to immunity is unknown, a number of observations suggest that this type of response does atleast correlate with inflammatory activity in the lesions of leprosy patients and hence the regulation of proliferative response is of great relevence[4,5].

In the present study PBMNC of the tuberculosis patients who showed normal responses to PHA showed decreased proliferative response to PMA and Con-A. The decrease in Con-A response was statistically significant. This decreased response is not due to toxicity because the same concentration of Con-A induced proliferative response in PBMNC of normal volunteers cultured *in vitro.* Reasons for the preferential reactivity to one or the other of these mitogens is not known. In man,

Con-A reactivity has been shown to be more sensitive to macrophage T cell interactions than PHA reactivity. Then it probably means that the lower response to Con-A in patients with tuberculosis reflects some abnormality at the level of macrophage. Ellner *et al* [7] showed that depletion of adherent cells enhance *in vitro* responsiveness of T lymphocyte to tuberculin thus demonstrating the presence of adherent suppressor cells.

Delaying the addition of antigen[1] or Con-A[8] has been shown to increase proliferative response. Bahr *et al* [9] showed that the proliferative response of PBMNC from tuberculoid (TT) and borderline leprosy (BL) cases and normal subjects were enhanced by delaying the addition of antigens to the LTT system which suggested a role for short lived suppressor cells. In our studies, the increase after delayed addition of various mitogens and antigens was seen only in a small percentage of volunteers and patients. Thus there was no evidence for the presence of specific regulator cells of short lived nature *in vitro.*

From the data it is difficult to conclude whether the responses from normal individuals to antigens of *M. tuberculosis* and other mycobacterial strains represent responses to common antigens of mycobacteria and/or represent true sensitization with individual strains. The high prevalence of environmental mycobacteria in this area increases the complexity of the problem[10].

Addition of indomethacin enhanced antigen induced proliferation in all the four normal individuals and in two patients. All the mycobacterial antigens showed the same effect. But when the basal antigen induced proliferation was low, indomethacin could not enhance it. The absence of this normal regulatory mechanism from the PBMNC of certain tuberculosis patients could be either due to failure of the antigens to induce synthesis or release of prostagladin E_2 (PGE_2) or the absence of target calls upon which the PG acts. It was also further observed that indomethacin failed to cause enhancement of response of non-adherent cells. This finding is consistent with the reported view that ceils of the monocyte/macrophage series are the main source of PGE-2 in vitro [8,11].

The hyporesponsiveness of cells from certain patients is not due to excessive PGs *in vitro* and there is no difference in the pattern of response to indomethacin among the various mycobacterial antigens tasted.

In costimulation experiments, inhibition of Con-A responses were not uniform and frequently, enhancement rather than inhibition was observed. This was observed with all three doses of Con-A. There was no specific pattern with any of the mycobacterialantigens used. Similar observations have been reported by Bahr *et al* who used *M. tuberculosis, M. kansasii, M. leprae, M. vaccae* and *M. nonchromogenicum*

with and without Con-A in their lymphoproliferative assay using PBMNC from normal individuals.

Costimulation of PBMNC by various mycobacterial antigens along with *M. tuberculosis* $H_{37}Rv$ revealed that the proliferation responses of PBMNC of both tuberculosis patients and volunteers to $H_{37}Rv$ were suppressed by the various environmental mycobacteria used. Therefore, any suppressive effect of these antigens on the cell mediated immunity of tuberculosis patients may not depend on an enhanced susceptibility of their PBMNC to these antigens. The reasons for this effect may be antigenic activity of the common mycobacterial components such as the cell wall glycopeptide or peptidoglycolipid and/or may be mediated by suppressor cells triggered by the shared antigens. This observation is strengthened by the study of Wadee *et al* showing au extractable lipid rich component of whole organisms involved in suppression.[12]

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