

## Evidence for the presence of *M. leprae* reactive T lymphocytes in patients with lepromatous leprosy

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### SUMMARY

Evidence for the presence of *Mycobacterium leprae* reactive T cells in many lepromatous leprosy (LL) patients was obtained using *in vitro* antigen-induced lymphoproliferative responses. (1) Co-cultures of T enriched cells from LL patients when combined with 2 h adherent cells (AC) from HLA-D compatible tuberculoid leprosy individuals showed significant levels of <sup>3</sup>H-thymidine incorporation in the presence of soluble and integral *M. leprae* antigens. (2) More interestingly, autologous T cell + AC co-cultures also showed significant improvement in antigen-induced lymphoproliferation in nine of 16 lepromatous patients. Insignificant improvement was observed in similar co-cultures of tuberculoid leprosy patients. (3) Addition of exogenous, purified human interleukin-2 (IL-2) to antigen stimulated PBMC from some lepromatous patients showed the best improvement in terms of overall <sup>3</sup>H-thymidine incorporation, indicating that lepromatous patients possess T cells which can differentiate to an IL-2 responsive state. Significantly, the level of proliferation varied within the group. A proportion of clinically similar lepromatous patients failed to show improvement by any of the above methods.

**Keywords** leprosy *Mycobacterium leprae* accessory cells interleukin-2

### INTRODUCTION

The antigen specific immunological unresponsiveness associated with lepromatous leprosy (LL) has been a challenging problem. Our previous studies investigating the mechanisms responsible for this anergy have indicated that suppressor T cell activity is generated as part of a well regulated immune response to the natural *Mycobacterium leprae* infection in man (Nath *et al.*, 1979, 1980; Nath & Singh, 1980). Lack of suppression in LL patients as demonstrated by us as well as others (reviewed by Nath, 1983) would preclude attributing a pathogenic role to the suppressor T cell subset. More significantly, monocyte rich adherent cells from lepromatous individuals have been shown to suppress *in vitro* proliferative responses of T cells from tuberculoid patients through the release of soluble factors (Sathish *et al.*, 1983) and by cell lysates (Salgame, Mahadevan & Antia, 1983). The present study was therefore undertaken to investigate whether modulation of *in vitro* responses would overcome the active suppression mediated by monocyte released factors.

Our results indicate that a proportion of LL patients possess *M. leprae* reactive T cells which can be induced to proliferate under defined conditions in the presence of (i) other HLA-D defined adherent cells (AC) from responder individuals, (ii) *in vitro* modulated autologous AC and (iii) exogenous human IL-2.

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## MATERIALS AND METHODS

**Patients.** A total of 74 LL and tuberculoid leprosy (TT) patients and three healthy contacts were studied. (i) Of these, 10, from Gandhi Memorial Leprosy Foundation, Wardha, were selected on the basis of HLA-D compatibility between paired individuals (sibling and non-sibling) with discordant leprosy (Table 1). The HLA data was kindly provided by J. J. van Rood, Department of Immunohaematology, Academisch Zeikenhuis, Leiden and N. K. Mehra, Department of Anatomy, All-India Institute of Medical Sciences, New Delhi, and formed part of earlier reports (DeVries *et al.*, 1980). These patients had received 50–100 mg daily dapsone for 2–6 years prior to testing. The contacts showed no evidence of clinical disease. (ii) The other patients were from All-India Institute of Medical Sciences and Safdarjang Hospital, New Delhi, and consisted of 30 untreated polar LL patients, nine untreated borderline lepromatous (BL) patients, 18 polar TT patients, four borderline tuberculoid (BT) patients and also six LL patients who had been treated for 1–5 years with 50–100 mg dapsone daily. All patients were graded on the basis of the Ridley–Jopling classification (Ridley & Jopling, 1966), supported by bacillary smears, histopathology of skin lesions and the level of *M. leprae*-induced lymphocyte transformation in peripheral blood mononuclear cell (PBMC) cultures. The lymphoproliferative responses were recorded as stimulation indices (mean counts per minute of cultures with antigen divided by the mean counts per minute of cultures without antigen) given as a percentage. Stimulation levels of  $\leq 100\%$  were consistently observed in PBMC cultures of lepromatous individuals, whereas tuberculoid patients and healthy contacts had antigen induced responses which ranged from 140–1,430%.

**Characterization of cells.** T cells were identified by OKT3, a pan-T cell monoclonal antibody using indirect immunofluorescence. Ficoll-hypaque (Pharmacia, Uppsala, Sweden) purified lymphocytes ( $10^6$ ) were incubated with 50  $\mu$ l of 1:50 dilution of OKT3 for 45 min at 4°C, washed three times with Hank's balanced salt solution (HBSS; Gibco Biocult, Paisley, UK), incubated for a further 45 min with 1:60 anti-mouse fluorescein isothiocyanate conjugated F(ab)<sub>2</sub> fraction (New England Nuclear Corp., Boston, Massachusetts, USA), washed three more times and suspended in 10% glycerol-phosphate-buffered saline (pH 7.2). B cells were identified by the presence of surface immunoglobulin (Ig) by direct immunofluorescence with 1:50 fluorescein isothiocyanate conjugated rabbit anti-human Ig (Cappel Laboratories, Cochranville, Pennsylvania, USA). Fluorescence was visualised by epi-illumination with an HBO 50 lamp (Carl Zeiss, Oberkochen, FRG). Monocytes were identified by (a) the presence of non-specific esterase using  $\alpha$ -naphthyl acetate as

**Table 1.** HLA haplotypes of donors of T cells and AC in co-cultures experiments depicted in Figs. 1 and 2

Expt.	Donor status	Cells	HLA haplotypes										
Fig. 1	(1)	TT	T cells	A2	BW40.2	W6	DW2/	A2	BW40.2	W6	DW2		
		Healthy contact	AC	A2	BW40.2	W6	DW2/	A2	BW40.2	W6	DW2		
	(2)	TT	T cells	A28	BW51	W4	CW6	DRW2/	AW33	BW53	W4	CW5	DRW2
		Healthy contact	AC	A28	BW51	W4	CW6	DRW2/	AW31	B22.1	W6	CW1	DRW6
	(3)	TT	T cells	A9	BW40	W6	DW2/	A11	B5	W4	DW2		
		Healthy contact	AC	A9	BW40	W6	DW2/	A11	B5	W4	DW2		
Fig. 2	(1)	LL	T cells	A2	B40	W6	DR2/	A28	B7	W6	DRW8		
		TT	AC	A2	BW40.2	W6	DW2	A2	BW40.2	W6	DW2		
		TT	AC	A2	B40	W6	DR2/	A2	B5	W4	DR2		
		TT	AC	A28	B51	W4	CW6	DRW2/	AW33	BW53	W4	CW5	DRW2
	(2)	LL	T cells	AW33	BW40	W6	DW2/	A11	B5	W4	DW2		
		TT	AC	AW33	BW40	W6	DW2/	A11	B5	W4	DW2		

per Yam, Li & Crosby (1971) and (b) phagocytosis of latex particles (0.3  $\mu\text{m}$  diameter; Sigma Chemical Co., St Louis, Missouri, USA). Mononuclear cells obtained as above were incubated with 50  $\mu\text{l}$  of 1% latex suspension at 37°C for 30 min.

*Stimulants.* Armadillo derived soluble and intact *M. leprae* bacilli (integral) was a kind gift from Dr R. J. W. Rees, National Institute for Medical Research, London, UK. Earlier studies on lymphoproliferation on cells from tuberculoid individuals had indicated 5  $\mu\text{g}$  protein per ml and  $5 \times 10^6$  *M. leprae* per ml to be optimal for soluble and integral antigens, respectively.

*Isolation of subpopulations of PBMC.* PBMC were isolated from venous blood of patients by Ficoll-hypaque centrifugation. PBMC were washed three times with HBSS and suspended in RPMI 1640 (GIBCO) buffered with 20 mM HEPES (*N*-2-hydroxy ethyl piperazine-*N'*-2-ethane sulphonic acid, GIBCO) and supplemented with 100 u penicillin/ml and 100  $\mu\text{g}$  of streptomycin/ml and 10% pooled AB serum (complete medium).

*Adherent (AC) and non-adherent cells (NAC).* Adherent cells were isolated from PBMC on 24 well tissue culture plates (Flow Laboratories, Irvine, UK) which had been pre-treated with fetal calf serum (FCS) (GIBCO). PBMC ( $10^6$  per well) were incubated at 37°C for 2 h. The NAC were removed by repeated pipetting and washing with pre-warmed HBSS. The adherent cells were recovered by incubating the wells with RPMI + 10% FCS containing 12 mM xylocaine (Astra-IDL, Bangalore, India) for 30–45 min followed by vigorous pipetting. In experiments where antigen pulsed monocytes were used, 250  $\mu\text{l}$  of the soluble *M. leprae* antigen were added to the adherent cells derived from  $1 \times 10^6$  PBMC and incubated overnight.

*Nylon wool column purified cells (NWC).* Two millilitre plastic sterile syringes packed with 40 mg of teased prewashed nylon wool fibres (Leuko-pak; Fenwall Laboratories, Illinois, USA) were freshly incubated with warm RPMI + 5% FCS for 1 h. Ten million NAC were added to each column and incubated vertically for 45 min at 37°C. The NWC was eluted with warm RPMI + 10% FCS through a 23 gauge needle. The eluted cells were washed and suspended in complete medium.

*Lymphoproliferation assay.* Cultures in quadruplicate, containing 100  $\mu\text{l}$  of  $10^5$  PBMC and  $10^5$  T enriched cells plus 25  $\mu\text{l}$  of 5, 10 and 20% AC were set up in round bottom microtitre plates (Nunc-Intermed, Denmark). They were incubated with and without antigen (25  $\mu\text{l}$ /well) for 6 days: cultures without antigen or AC served as controls. The cultures were harvested onto glass fibre discs by a semi-automatic harvester (PHD Cell Harvester, Cambridge, Massachusetts, USA) 16 h after the addition of 0.5  $\mu\text{Ci}$   $^3\text{H}$ -thymidine (specific activity 2 Ci/mmol, Amersham International, UK). The radioactivity was assessed in a LKB Rackbeta II 1215 Scintillation Counter using toluene based scintillation fluid.

*Human T cell growth factor (TCGF/IL-2).* TCGF/IL-2 (Morgan, Ruscetti & Gallo, 1976; Gillis et al., 1978) was prepared from a human cell line (Jurkat 4) kindly supplied by Dr Max H. Schreier, Basel Institute of Immunology, Switzerland. Batches of 30 ml cultures with  $10^6$  cells per ml were set up in RPMI 1640 containing 10% FCS, and 1% phytohaemagglutinin-M (DIFCO), and maintained for 40 h at 37°C in humidified 5%  $\text{CO}_2$  and air. The supernatant containing IL-2 was collected by centrifugation of cells at 150g. Subsequently, 250 ml of supernatant was purified by precipitation with 85%  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was dissolved in 10 ml of 10 mM HEPES-buffered saline (pH 7.2), dialysed against the same buffer for 16–18 h and chromatographed on Sephadex G-100 (Pharmacia, Uppsala, Sweden). The post-albumin fractions containing T cell growth promoting activity were pooled, sterilized through 0.45  $\mu\text{m}$  filters (Millipore Corp., Bedford, Massachusetts) stored in aliquots at  $-70^\circ\text{C}$  and used in subsequent studies. This preparation migrated as a single band in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

IL-2 activity was assayed on Con A-induced murine lymphoblasts (Anderssen et al., 1979).  $10^6$ /ml of spleen cells from BALB/c mice in RPMI 1640 + 10% FCS were cultured in 24 well tissue culture plates (Linbro, Flow) in the presence of 2.5  $\mu\text{g}$ /ml Con A (Pharmacia) at 37°C for 72 h. On the 3rd day, Con A activated blasts were washed three times with RPMI 1640 at 150g.  $2 \times 10^4$  blasts (100  $\mu\text{l}$  per well) were cultured for a further 2 days, in 96 well microtitre plates with 100  $\mu\text{l}$  of various dilutions of IL-2 ranging from 1:5 to 1:1,000. At 44 h, 25  $\mu\text{l}$  of 0.5  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (sp. act. 2 Ci/mmol, Amersham) was added to the wells and the cultures harvested 4 h later as described earlier. At 1:5 dilution of IL-2, the mean ct/min  $\pm$  s.d. obtained was  $20,498 \pm 4,102$ .

Preliminary experiments with further dilutions of IL-2 were conducted on human PBMC to rule

out possible mitogenic effects. Since variable mitogenic effects were noted in different individuals, all patients were tested with 1:20, 1:40 and 1:80 concentrations of IL-2.

*Statistical analysis.* Statistical analysis was performed using the Mann-Whitney U-test (Siegel, 1956).

## RESULTS

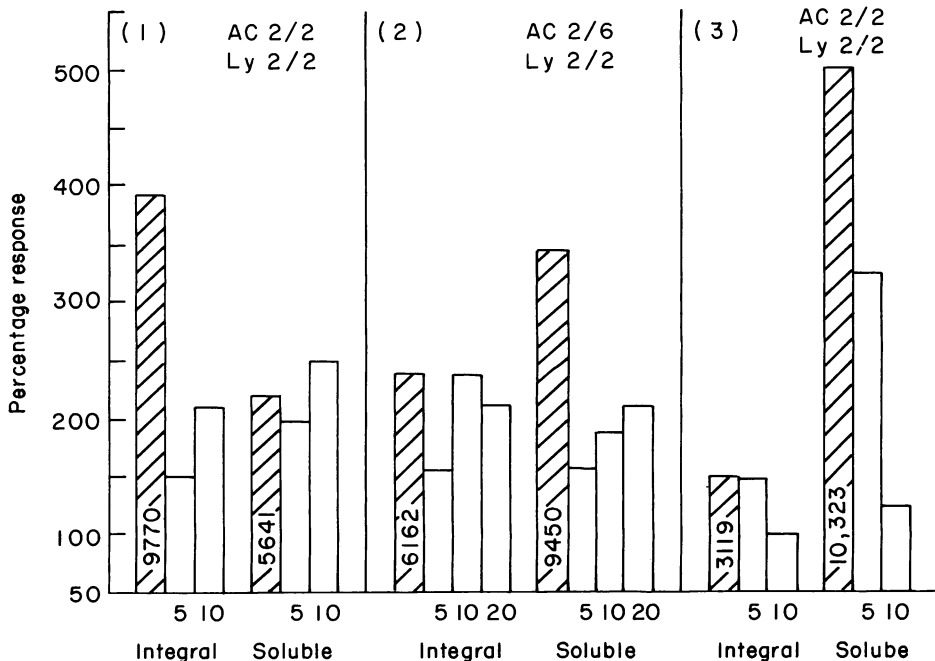
### *Effect of AC on M. leprae induced lymphoproliferation of T enriched cells from leprosy patients*

Twenty-one LL and 11 TT patients and three healthy contacts were studied using integral and soluble armadillo derived *M. leprae* antigen-induced lymphoproliferation of PBMC and T cell + AC co-cultures. Non-adherent cells and nylon wool purified cells derived from PBMC of TT and LL patients showed similar numbers of OKT3+ cells, B cells and non-specific esterase positive monocytes (data not given).

### *Lymphoproliferation in HLA-D defined T cell + AC co-cultures*

Variable levels of antigen-induced lymphoproliferation was observed when nylon wool purified T cells derived from three TT patients were combined with 2 h adherent cells from HLA-D compatible healthy responder siblings (Table 1 & Fig. 1). Whereas 1 and 2% AC were inadequate, 5, 10, 20% AC were able to reconstitute the proliferative responses to integral and soluble antigens.

More importantly, significant proliferation ( $P < 0.05$ ) to *M. leprae* antigens was also obtained in similar co-cultures where T cells derived from two LL patients were combined with AC from TT individuals who shared one or both HLA-D haplotypes. Interestingly, in one experiment, where AC



**Fig. 1.** Reconstitution of *in vitro* antigen-induced lymphoproliferative responses of three co-cultures wherein  $10^5$  nylon wool purified T enriched cells (Ly) from TT patients were combined with 5, 10 and 20% 2 h AC (abscissa) from HLA compatible healthy responder siblings and stimulated with integral and soluble *M. leprae* antigens. HLA and HLA-D haplotypes of donors of both sets of cells are given in Table 1 and on top of the figures, respectively. Open and hatched bars indicate responses of co-cultures and PBMC cultures of TT patients, respectively. Mean ct/min of PBMC is given inside the shaded bars. The s.d. was  $< 25\%$ . Percentage response = (mean ct/min of cultures with antigen/mean ct/min of cultures alone)  $\times 100$ .

were obtained from three donors all of whom had HLA-D haplotype 2/2, successful stimulation was possible with only one non-sibling donor (Fig. 2).

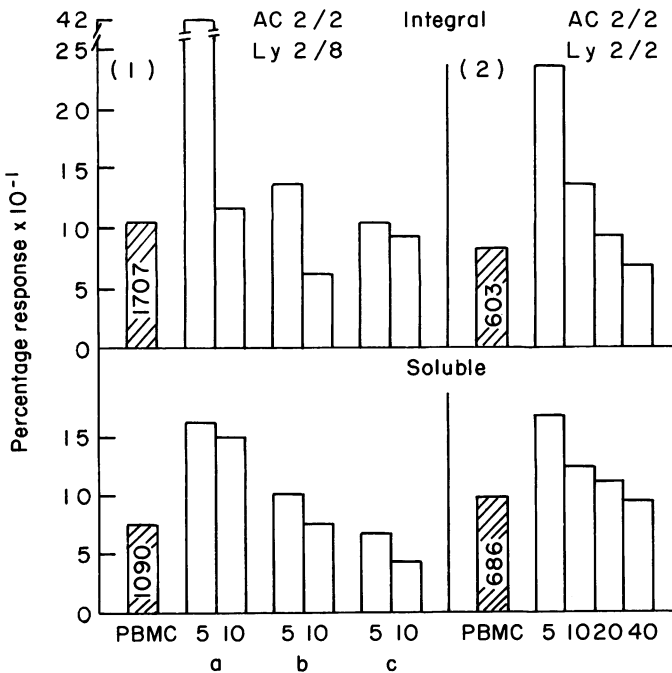
Supernatants derived from AC cultures of eight TT patients were unable to support T cell proliferation when added to PBMC, nylon wool separated T cells and NAC from LL and TT patients.

#### Lymphoproliferation in autologous T cell + AC co-cultures

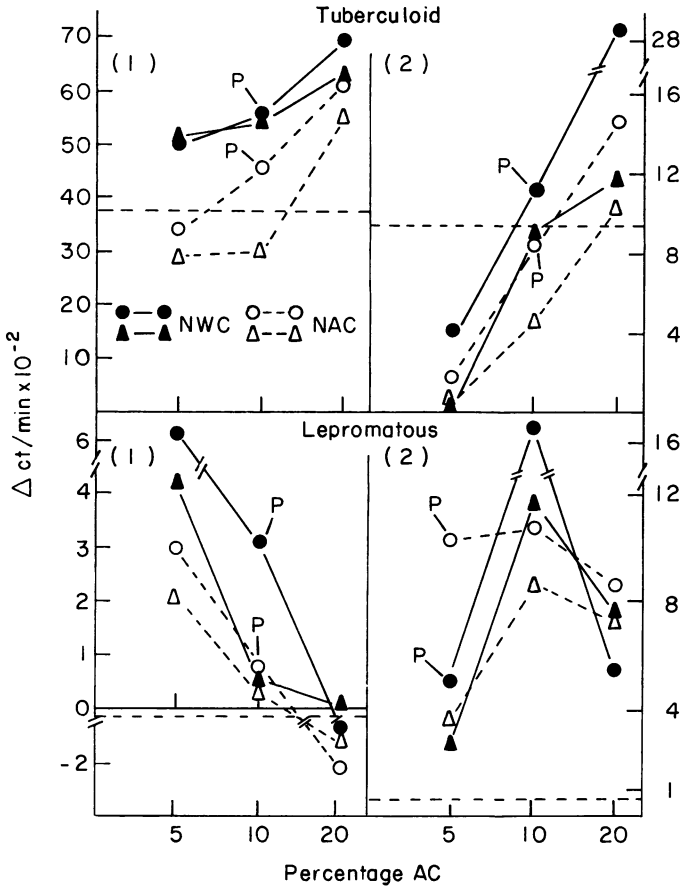
In order to evaluate whether adherent cells from LL individuals could be induced to support *in vitro* lymphoproliferation, co-cultures similar to the above were undertaken using autologous T cell + AC combinations. T cells were obtained from NAC fraction as well as after passage through nylon wool columns. AC were used as such or after 18 h prior 'pulsing' with soluble *M. leprae* antigen. Both types of AC were added to T cells at the same time and those with 'pulsed' AC were left without further addition of antigen.

Fig. 3 shows representative data obtained. In accordance with the earlier results using HLA-D defined co-cultures (Nath *et al.*, 1980; Sathish *et al.*, 1983), it was noted that 10 and 20% AC gave the best T cell stimulation in TT. It was consistently observed that 'pulsed' AC were better than non-pulsed AC and nylon wool fractionated cells were superior to NAC for antigen-induced proliferation (Fig. 3).

Cell combinations from LL patients showed a requirement for similar culture conditions with the added proviso that the concentration of AC needed to produce proliferation in most cultures varied between 5 and 10%. Some individuals showed a graded inhibition of proliferation in the presence of increasing concentrations of AC (Fig. 3). Occasionally, 2% AC produced maximal stimulation.



**Fig. 2.** Stimulation of *in vitro* antigen-induced lymphoproliferation in four co-cultures wherein  $10^5$  nylon wool purified T enriched cells (Ly) from two LL patients were combined with 5, 10, 20, 40% 2 h AC (abscissa) from four TT patients in the presence of integral and soluble *M. leprae* antigens. HLA and HLA-D haplotypes of donors of both sets of cells are given in Table 1 and on top of the figures, respectively. Open bars indicate co-cultures whereas hatched bars refer to PBMC cultures of lepromatous patients with mean ct/min values shown inside the bars. The s.d. was <15%. (a), (c) refer to non-siblings and (b) to sibling donor of AC. Percentage response is same as in legend to Fig. 1.



**Fig. 3.** Representative autologous co-cultures showing the pattern of soluble *M. leprae* antigen-induced *in vitro* lymphoproliferative responses of two TT and two LL individuals. One hundred thousand NAC and NWC T cells were combined with 5, 10, 20% autologous AC (abscissa) which had been used as such or after 18 h prior 'pulsing' (P) with soluble *M. leprae*. --- = peripheral blood mononuclear cells + *M. leprae* antigens.  $\Delta$  ct/min = mean ct/min of antigen stimulated cultures - mean ct/min of cultures without antigen.

When maximal  $\Delta$  ct/min obtained in the co-cultures was compared with that of parallel PBMC, LL patients fell into two categories (Table 2). (i) Moderate to marked improvement in lymphoproliferation was observed in co-cultures from nine LL/BL patients. (ii) No significant change was observed in seven other LL patients. The BT/TT group showed statistically insignificant increases in proliferation in co-cultures as compared to PBMC (Table 2).

#### *Effect of IL-2 on M. leprae-induced lymphoproliferation of LL patients*

TCGF/IL-2 obtained from a human cell line (JR4) was added to PBMC cultures of LL/BL patients at a final concentration of 1/20, 1/40 and 1/80. In general, 1/40 gave the least mitogenic effect and the maximal proliferation (Table 3). Of the 30 patients studied, 21 showed enhanced lymphoproliferation in antigen stimulated cultures with IL-2. The level of proliferation varied markedly within the group, mean ct/min  $\pm$  s.d. of cultures with antigen + IL-2 ranged from  $1,852 \pm 393$  to  $27,168 \pm 1,801$ . Representative data on 15 patients is given in Table 3. This variability could not be attributed to any differences in the clinical pattern of disease. Significantly, nine similar LL patients failed to show IL-2-dependent improvement in lymphoproliferation.

## DISCUSSION

The current results indicate that many LL patients can be shown to possess antigen reactive T cells

**Table 2.** Soluble *M. leprae* antigen-induced responses of nylon wool column purified T cells reconstituted with autologous 'pulsed' AC from leprosy patients

Expt. No.	Diagnosis	<sup>3</sup> H-thymidine incorporation ( $\Delta$ ct/min)*		Significance† PBMC vs T+AC
		PBMC	Maximal response in T+AC	
1	LL	-62	1,581 (5)‡	<0.001
2	LL	56	1,177 (10)	
3	LL	10	506 (5)	
4	LL	112	1,690 (5)	
5	BL	-3	1,088 (10)	
6	LL	93	1,511 (5)	
7	BL	257	616 (5)	
8	LL	-206	409 (5)	
9	LL	-83	1,400 (10)	
10	BL	-40	40 (5)	NS
11	LL	71	83 (5)	
12	LL	-110	16 (5)	
13	LL	-35	132 (5)	
14	BL	65	95 (5)	
15	LL	29	82 (5)	
16	LL	-71	123 (5)	
17	TT	1,476	2,106 (10)	NS
18	TT	2,145	3,960 (20)	
19	TT	397	993 (20)	
20	BT	3,744	6,896 (10)	
21	BT	3,979	7,850 (20)	
22	TT	3,155	4,972 (20)	

\* $\Delta$  ct/min = mean ct/min antigen - mean ct/min control.

† Statistical significance was calculated using the Mann-Whitney U-test.

‡ Values in parenthesis refers to concentration of adherent cells giving maximal responses.

which are capable of *in vitro* lymphoproliferation in the presence of integral and soluble *M. leprae* antigens.

In accordance with earlier studies (Nath *et al.*, 1980), it was noted that adherent cells from TT individuals when combined with T cells of HLA-D compatible LL patients stimulated antigen-induced lymphoproliferation in hitherto non-responsive individuals. More interestingly, AC derived from LL patients were also able to stimulate autologous T cells in the presence of antigen. Under defined conditions significant improvement in lymphoproliferation ( $P < 0.001$ ) was observed in 56% of the LL individuals studied. In terms of mean ct/min the increment in the stimulated co-cultures was in the low to moderate range (409-1,690). These studies indicate that both antigen reactive T cells and effective antigen presenting accessory cells are present in LL. Our earlier studies showing that suppressive factors are released by monocyte rich adherent cell cultures (Sathish *et al.*, 1983) would suggest that the final expression of the immune responses *in vivo* and *in vitro*, as well as the variability in the degree of stimulation observed in autologous cocultures may be due to the relative proportion of immunostimulatory and immunosuppressive cells within the AC populations. Subsets of adherent cells with these opposing functions have been detected in various experimental situations (Lee & Berry, 1977; Wing *et al.*, 1977; Harrington-Fowler & Wilder, 1982; Olds *et al.*, 1983). Moreover, adherent dendritic cells with accessory cell function in various

**Table 3.** Representative data on the effect of IL-2 on *M. leprae*-induced lymphoproliferative responses of LL patients

No.	Mean ct/min				IL-2+ML
	PBMC alone	+IL-2	+ML	+IL-2+ML‡	IL-2
1*	378	379	443	27,168	71·6
2	143	173	121	8,888	51·3
3	1,224	455	2,355	11,381	25·0
4	1,157	1,233	1,350	20,970	17·0
5	523	615	626	10,488	17·0
6†	644	366	1,014	3,681	10·1
7	673	778	545	4,738	6·1
8	180	390	223	1,852	4·8
9	599	527	379	1,908	3·6
10	658	733	590	1,959	2·7
11	820	10,127	763	19,152	1·9
12	155	155	173	141§	1
13	195	162	167	147§	1
14	219	167	190	147§	1
15	198	1,367	365	1,223§	0·9

Standard deviation  $\leq 20\%$  of mean ct/min.

IL-2 dilution was 1:40 except in \* and † where 1:20 and 1:80, respectively, was most effective.

‡ *P* value ranging from  $\leq 0\cdot05$  to  $\leq 0\cdot01$  when compared to IL-2 alone.

§ Not significant.

lymphoproliferative assays have been shown to be present in the peripheral blood of man (van Voorhis *et al.*, 1983).

Further evidence for the presence of antigen reactive T cells in LL was obtained in experiments using exogenous, purified IL-2. In conformity with other studies (Hargewoin *et al.*, 1983) PBMC from 21 of 30 LL patients showed moderate to high antigen-induced proliferative responses in the presence of non-mitogenic concentrations of IL-2. It would appear therefore that LL individuals have T cells which can differentiate to an IL-2 responsive state on interaction with antigen. In the natural state, the unresponsiveness may be related to the inhibition of further expansion of these cells by active suppressive mechanisms. The monocyte released soluble factor reported earlier by us may be responsible for this suppression. The fact that suppression can be overcome by exogenous IL-2 suggests that this suppression occurs before IL-2 production or utilization. Interestingly, the suppression may dictate the final response. One-third of the patients failed to show IL-2-mediated improvement in antigen-induced lymphoproliferation.

One of the significant features of the above as well as earlier studies (Hargewoin *et al.*, 1983) is that a proportion of clinically similar LL patients fail to show improvement by any of the above methods. The presently available studies on the potential candidate vaccines also indicate that only 55–65% of lepromatous individuals responded by a positive skin test or bacterial clearance (Convit *et al.*, 1979; Deo *et al.*, 1981). A discrimination between the potentially responsive and the non-responsive LL patients is necessary not only for the understanding of the disease but also for the future of a vaccine against leprosy.

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