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The suppressive effect of *M. leprae* on the *in vitro* proliferative responses of lymphocytes from patients with leprosy

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SUMMARY

Peripheral blood lymphocytes from sixty leprosy patients and eight healthy contacts known to be responsive to *M. leprae*, were stimulated *in vitro* with concanavalin A (Con A) or PPD alone or in combination with autoclaved, whole *M. leprae*. Time kinetics and the percentage of inhibition induced by *M. leprae* differed in the two disease groups and contacts. Antigen-generated suppression of Con A-stimulated lymphocyte transformation was observed on day 4 in seventeen of twenty-one (80%) tuberculoid patients and six of seventeen (35·3%) untreated lepromatous patients. Healthy contacts and 53% lepromatous individuals showed enhanced Con A responses in the presence of antigen. On prolongation of antigen presence to 6 days, a marginal effect was noted in the tuberculoid group. In contrast, all healthy individuals and some lepromatous patients showed increased inhibition of Con A responses. *M. leprae* antigens showed uniform inhibition of PPD-induced ^{3}H-thymidine incorporation in leprosy patients and healthy contacts.

INTRODUCTION

Over the last decade, leprosy has emerged as a complex and intriguing problem in immunology. Infection with *Mycobacterium leprae* is manifested as a clinical and immunological spectrum ranging from the localized, high-resistant tuberculoid to bacilliferous, low-resistant lepromatous leprosy (Ridley & Jopling, 1966; Myrvang *et al.*, 1973). The diverse immunological aberrations observed in leprosy have been extensively reviewed (Turk & Bryceson, 1971; Godal *et al.*, 1974; Godal, 1978). Lepromatous patients consistently fail to react to contact sensitizing agents, ubiquitous and *M. leprae*-specific skin test antigens and show reduced *in vitro* responses to T cell mitogens and *M. leprae* antigens. On the other hand, humoral responses appear normal or enhanced. Increased B cell numbers, higher levels of anti-mycobacterial and anti-*M. leprae*-specific antibodies have been observed in these patients. The general pattern that emerges from the various studies in lepromatous leprosy is that of an imbalance between humoral and cellular immune responses along with an inability to eliminate the causative organism. In contrast, patients with tuberculoid leprosy maintain integrity of T cell functions, have low levels of anti-mycobacterial antibodies and successfully eliminate *M. leprae* from the tissues (Turk & Bryceson, 1971; Godal, 1978).

The precise mechanisms responsible for the immunological disturbances noted in leprosy remain obscure. In recent years, immunoregulatory control systems have been demonstrated in experimental animals. Of particular interest has been the discovery of a subset of T cells which have

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suppressor functions and regulate immune responses through negative feedback mechanisms (Gershon, 1974). In man, suppressor T cells have been identified by surface markers (Moretta *et al.*, 1976). *In vitro* induction of suppressor activity by antigens, mitogens and allogeneic cells (Shou, Schwartz & Good, 1976; Sakane & Green, 1977; Hirschberg & Thorsby, 1977) has also been observed.

Perturbations of suppressor activity have been implicated in several diseases (Abdou *et al.*, 1976; Fauci *et al.*, 1978; Ellner, 1978; Pelley & Warren, 1978). Recent evidence indicates that mycobacterial infections in mice result in immunoregulatory disturbances (Alexander, 1978; Bullock, Carlson & Gershon, 1979). With a view to assessing the role of suppressor T cells in human leprosy, we had enumerated the numbers of T cells bearing Fc receptors for IgG (Singh & Nath, 1980) and studied the *in vitro* inducibility of suppressor activity by concanavalin A (Con A) in patients with tuberculoid and lepromatous leprosy (Nath *et al.*, 1979).

In the present study we have explored the influence of *M. leprae* antigens on the *in vitro* proliferation of lymphocytes from leprosy patients and healthy contacts. Antigen-induced suppression of Con A/PPD-stimulated lymphocyte DNA synthesis has been assessed by the inhibition of tritiated thymidine uptake in cultures simultaneously stimulated with *M. leprae* and Con A/PPD.

MATERIALS AND METHODS

Test subjects. Sixty leprosy patients attending the leprosy clinics of Lok Nayak Jayaprakash Narayan Hospital were included in the study. They were selected on the basis of Ridley–Jopling classification (1966) after clinical assessment, histopathological, slit-smear examination of the skin for bacilli and *in vitro* lymphocyte transformation to *M. leprae*. Thirty-five LL and two BL patients were broadly classified as lepromatous (L). Twenty TT and three BT patients were grouped as tuberculoid (T). Two TT and three LL patients had received 600 mg dapsone for 3–5 years and 6–12 months respectively. All other patients were untreated and did not show any reactions at the time of examination. Sex distribution and nutritional status of the two groups of patients were similar.

The control group consisted of eight healthy individuals in contact with leprosy patients and *M. leprae* antigens for 2–5 years. These individuals showed significant *in vitro* lymphocyte transformation to *M. leprae*, with stimulation indices (SI) ranging from 3 to 5 (SI = c.p.m. of cultures with *M. leprae*/c.p.m. of cultures without antigen).

Stimulants. Concanavalin A (Con A; Pharmacia Laboratories) and PPD (Ministry of Agriculture, Central Veterinary Laboratory, Weybridge, UK) were used at optimum stimulatory doses of 300 and 50 μg per ml respectively. Whole, washed, autoclaved *M. leprae* was used at a concentration of 5×10^6/ml which was found to give optimal stimulation in our system.

In vitro lymphocyte transformation test. Briefly, mononuclear cells were separated from sterile heparinized blood (10 iu preservative-free heparin per ml; Upjohn Co.) by density gradient centrifugation as described by Böyum (1968) (Lymphoprep, Nyegaard Co., Oslo) at 400 ***g*** at room temperature for 30 min. The cells collected at the interphase were washed three times in Eagle's minimum essential medium (MEM; GIBCO BIOCULT) and resuspended in RPMI 1640 at 1×10^6 cells per ml supplemented with 10% pooled AB serum, streptomycin and penicillin. One hundred thousand cells in 100 μl were cultured in plastic microtitre plates (Nunc Intermed, Denmark) in quadruplicate with or without 25 μl of antigen in the presence of 5% CO_2 and air. Fourteen to sixteen hours prior to harvesting 1 μCi of tritiated thymidine (^{3}H-TdR; specific activity 2 Ci/mmol; Bhabha Atomic Research Centre, Trombay) was added to each culture. The antigen cultures were harvested on day 6 on to glass fibre paper, dried and counted in a liquid scintillation counter.

Suppressor cell assay. The effect of *M. leprae* on Con A- and PPD-stimulated lymphocyte transformation was studied. Mononuclear cells were cultured as described in the preceding section in microtitre plates with 10^5 cells per well. Replicate batches of cells were exposed to: (i) medium alone, (ii) Con A, (iii) PPD, (iv) *M. leprae*, (v) a mixture of Con A and *M. leprae* or (vi) a mixture of PPD and *M. leprae*. Con A was added 3 days prior to harvesting whereas *M. leprae* and PPD were present throughout the culture period. After time-kinetics of optimum period had been assessed (Fig. 1) subsequent cultures were harvested on days 4 and 6. Suppression of the *in vitro* responses

was calculated as follows and per cent inhibition above 10% was found to be significant ($P<0.05$). Mean counts per minute of replicate cultures were calculated.

(i) $$\text{Per cent Con A response} = \frac{\text{c.p.m. } M.\ leprae \text{ with Con A}}{\text{c.p.m. with Con A alone}} \times 100;$$

(ii) $$\text{Per cent inhibition of Con A response} = 100 - \frac{\text{c.p.m. } M.\ leprae \text{ with Con A}}{\text{c.p.m. Con A} + \text{c.p.m. } M.\ leprae} \times 100;$$

(iii) $$\text{Per cent inhibition of PPD response} = \text{(a) } 100 - \frac{\text{c.p.m. } M.\ leprae \text{ with PPD}}{\text{c.p.m. PPD alone}} \times 100;$$

$$\text{(b) } 100 - \frac{\text{c.p.m. } M.\ leprae \text{ with PPD}}{\text{c.p.m. PPD} + \text{c.p.m. } M.\ leprae} \times 100.$$

Statistical analysis. The Mann-Whitney two-tailed test was used (Siegal, 1956).

RESULTS

Lymphocyte transformation to M. leprae antigens

The baseline lymphocyte response to *M. leprae* antigens in the individuals included in the study is given in Table 1. Eleven lepromatous patients showed inhibition of ^{3}H-thymidine incorporation in the presence of *M. leprae* antigens as indicated by a stimulation index (SI) of less than 1.

Time-kinetics of suppression of Con A responses by M. leprae antigens

In order to assess the time-kinetics of *M. leprae*-induced *in vitro* suppression, cultures were terminated from days 1 to 6 in three experiments. Except for cultures terminated on days 1 and 2, Con A was added 3 days prior to harvest whereas *M. leprae* was present throughout the culture period. This was done to assess the influence of *M. leprae* at the optimal period of mitogenic stimulation. It may be observed from Fig. 1a that the three tuberculoid patients studied showed uniform suppressor effects when *M. leprae* had been in culture for 4 days. A further increase in suppression was noted on days 5 and 6 in two of the patients. Lymphocytes from the three untreated lepromatous patients studied showed variable effects. Significant suppression was observed in two of the patients from day 5 onwards (Fig. 1b).

These experiments indicated that (i) a time period of 4 days was required for *M. leprae*-induced suppression, (ii) at the time of optimal antigenic stimulation (i.e. day 6) suppression was maximal in some leprosy patients, and (iii) tuberculoid and lepromatous patients showed different patterns of *M. leprae*-induced inhibition of Con A responses.

Table 1. Lymphocyte responses to whole, autoclaved *M. leprae* bacilli in healthy contacts and leprosy patients

	Stimulation index	
Subject	Median	Range
Healthy contacts (8)	3·8	3–5
Lepromatous (37)	1·02	0·89–1·2
Tuberculoid (23)	4·6	2·5–7·5

Figures in parentheses indicate number of patients studied.

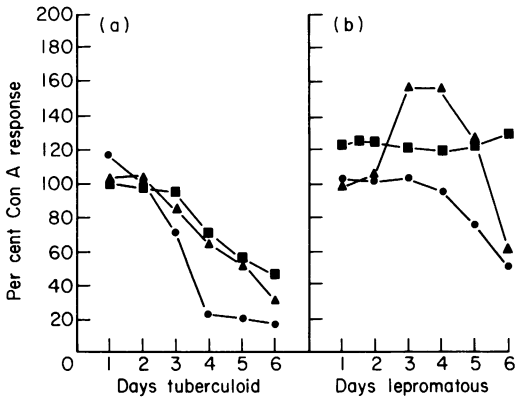

Fig. 1. Time kinetics of lymphocyte responses of tuberculoid and lepromatous patients to simultaneous stimulation with *M. leprae* and concanavalin A. Each line represents the same batch of lymphocytes incubated for varying time periods with *M. leprae*. Con A was added 3 days prior to harvest in cultures harvested on days 4–6.

Effect of M. leprae antigens on in vitro Con A responses of leprosy patients and healthy contacts

In view of the above findings subsequent cultures were harvested on days 4 and 6. The overall pattern of inhibition of *in vitro* mitogenic responses in the various categories of individuals is shown in Fig. 2 and Table 2. Individual variability in the degree of suppression was noted in each group and this was reflected in the size of standard deviation shown in Table 2. The per cent inhibition and per cent enhancement (+) for the group have been indicated as median values and non-parametric statistical methods were used.

Day 4. None of the healthy contacts showed significant inhibition (median + 15·1%). Four of eight individuals had enhanced Con A responses in the presence of *M. leprae*. In the tuberculoid group seventeen of twenty-one (80%) patients showed inhibition ranging from 15 to 79% (median

Table 2. Effect of *M. leprae* on *in vitro* Con A-induced lymphocyte transformation of leprosy patients and healthy contacts

	^{3}H-thymidine incorporation. Mean c.p.m. ± s.d. (median values)		
	Lepromatous	Tuberculoid	Healthy contacts
Day 4			
M. leprae	635·0 ± 451·7 (464)	889·0 ± 136·9 (708)	848·0 ± 404·5 (697)
Con A	8,578·0 ± 6,086·6 (6,709)	12,493·0 ± 1,660·0 (12,624)	26,686·0 ± 2,086·0 (24,496)
Con A + *M. leprae*	10,109·8 ± 9,394·0 (8,645)	9,061·0 ± 6,016·7 (9,180)	24,556·0 ± 8,725·0 (28,322)
Median % effect	+ 8·0	23·0	+ 15·1
Number of subjects	17	21	8
Day 6			
M. leprae	997·0 ± 976·7 (751)	2,259·0 ± 821 (1,292)	1,626·0 ± 874 (1,314)
Con A	4,276·0 ± 1,144·0 (1,131)	10,116·0 ± 4,829·0 (7,876)	9,109·0 ± 4,512 (8,171)
Con A + *M. leprae*	2,309·0 ± 2,337·0 (3,108)	6,978·0 ± 4,820·0 (6,904)	7,348·0 ± 4,000 (5,881)
Median % inhibition	15·5	26·5	31·5
Number of subjects	15	15	8

+ = % enhancement, − = % inhibition.

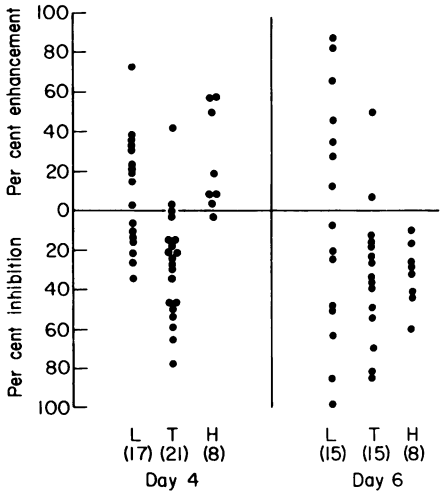

Fig. 2. The effect of *M. leprae* antigens on *in vitro* concanavalin A response of lymphocytes from lepromatous (L), tuberculoid (T) patients and healthy contacts (H). Cultures were terminated on days 4 and 6. Antigens were present throughout the culture period and Con A was added 3 days prior to harvest. Figures in parentheses indicate number of patients studied. Per cent inhibition was highly significant in the tuberculoid group as compared to healthy contacts ($P<0{\cdot}002$).

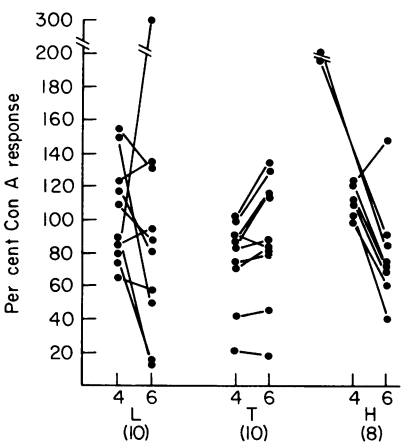

Fig. 3

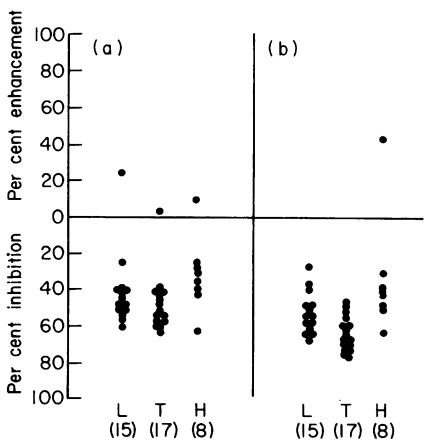

Fig. 4

Fig. 3. Percentage of Con A responses noted in the same batch of lymphocytes simultaneously stimulated with *M. leprae* antigens and Con A for 4 and 6 days. Increase in inhibition noted between the two time periods was highly significant in healthy contacts (H) ($P<0{\cdot}002$). Tuberculoid (T) patients showed marginal differences ($P<0{\cdot}1$). Four lepromatous patients showed increased inhibition on day 6.

Fig. 4. Percentage inhibition of PPD-induced *in vitro* lymphocyte responses in the presence of *M. leprae* antigens. Lepromatous (L), tuberculoid (T) patients and healthy contacts (H) showed significant inhibition ($P<0{\cdot}001$). Per cent inhibition = (a) $100-[$(c.p.m. *M. leprae* with PPD)/(c.p.m. PPD alone)$]\times 100$, (b) $100-[$(c.p.m. *M. leprae* with PPD)/(c.p.m. PPD + c.p.m. *M. leprae*)$]\times 100$.

Table 3. *M. leprae*-induced suppression on *in vitro* PPD-stimulated lymphocyte transformation of leprosy patients and healthy contacts

	^{3}H-thymidine incorporation. Mean c.p.m. ± s.d. (median)		
	Lepromatous	Tuberculoid	Healthy contacts
M. leprae	825·0 ± 238·4 (658)	3,162 ± 812·4 (1,324)	1,926·0 ± 860·0 (1,287)
PPD	9,300·0 ± 5,436·0 (7,806)	9,867·0 ± 2,310·0 (8,401)	10,968·0 ± 1,276·0 (9,261)
M. leprae + PPD	5,786·0 ± 3,826·0 (5,049)	4,826·0 ± 3,420·0 (4,065)	5,628·0 ± 3,126·0 (7,791)
Median % inhibition	48·0	55·5	34·0
Number of subjects	15	17	8

Per cent inhibition = 100 − [(c.p.m. *M. leprae* with PPD)/(c.p.m. PPD + c.p.m. *M. leprae*)] × 100.

23%). One patient showed enhancement. Suppression in the tuberculoid group was highly significant when compared with the responses of healthy controls ($P < 0·002$). Lepromatous patients showed greater variability in their proliferative responses. Six (35·3%) showed inhibition (range 10–35%) whereas nine showed enhancement of Con A responses (median + 8%).

Day 6. The *in vitro* suppressor effects due to *M. leprae* were more uniform in the three groups when antigenic stimulation was increased to 6 days. The healthy contacts showed reverse changes and had inhibition of mitogen responses ranging from 15–59% (median 31·5%). The increase in inhibition in the tuberculoid group was marginal ($P < 0·1$) whereas seven lepromatous individuals showed inhibition ranging from 20–98%. As a group the latter did not show statistically significant inhibition as many patients showed enhanced responses. Median inhibition in the lepromatous group was 15·5%.

In order to study the time-related changes in antigen-induced suppressor activity, 4- and 6-day cultures using the same batches of lymphocytes from individual subjects and patients were analysed further. Fig. 3 confirms the earlier observations. (i) Healthy contacts who showed enhanced responses on day 4 revealed suppression on day 6; (ii) six lepromatous patients showed increased inhibition on day 6 whereas (iii) tuberculoid patients did not show a significant change.

It would appear therefore that *in vitro* suppression was more consistently observed in tuberculoid patients and had different time-kinetics in the different groups of individuals studied. Enhanced responses were more frequently seen in the lepromatous group than in the tuberculoid patients.

M. leprae-induced suppression of in vitro PPD responses

The effect of *M. leprae* on *in vitro* lymphocyte transformation to PPD was studied on day 6 (Fig. 4, Table 3). *M. leprae* and PPD were added concurrently as the optimum period of stimulation for PPD in our hands was on day 6. Since it would be difficult to differentiate the effects of the two stimulants, the per cent inhibition of ^{3}H-TdR noted in cultures with simultaneous stimulation was expressed in relation to (i) c.p.m. in PPD cultures alone (Fig. 4a) and (ii) sum of c.p.m. in concurrent cultures with *M. leprae* and PPD (Fig. 4b).

By both criteria, it was observed that *in vitro* PPD responses were consistently and significantly inhibited by *M. leprae* antigens in both groups of patients and healthy contacts (Fig. 4 and Table 3). The per cent inhibition in the three groups of subjects showed less variability in the PPD responses as compared to the mitogen responses (Figs 2 and 4).

DISCUSSION

In spite of the extensive studies undertaken in leprosy patients over the last decade, the mechanism underlying the various immunological perturbations remains obscure. It has been difficult to

analyse which of the immunological phenomena are (i) directly responsible for the disease, (ii) appear secondary to antigenic load and (iii) are epiphenomena irrelevant to the causation or pathogenesis of the disease. Analysis is further hampered by the fact that many of the immunological parameters have been studied at particular points of time in a disease which is known to have a long incubation period. Thus, neither the time of onset of the disease nor the changing quantum of antigenic load can be assessed at the time of immunological testing. Moreover, *M. leprae* is known to have many antigenic components (Stanford *et al.*, 1975; Harboe *et al.*, 1977) and which of these stimulate or suppress the host responses is not known.

In the present study autoclaved, whole *M. leprae* bacilli were capable of inducing *in vitro* suppressor activity in leprosy patients and healthy contacts. The suppression induced was capable of inhibiting ^{3}H-TdR incorporation in lymphocytes stimulated by a T cell mitogen, Con A, and a 'recall' cross-reacting antigen, PPD. Three patterns of suppression emerged in tuberculoid, lepromatous patients and healthy contacts. (i) Tuberculoid leprosy patients showed consistent and significant suppression of *in vitro* Con A responses by day 4. This suppression persisted but was not significantly increased on further incubation with *M. leprae* to 6 days. Both the amount of inhibition and the number of patients showing inhibition was higher in this group than in lepromatous patients or healthy contacts. Moreover, enhancement of Con A and PPD responses by simultaneous stimulation with *M. leprae* was rare. (ii) Lepromatous patients showed variable Con A responses in the presence of *M. leprae* antigens. The number of patients showing suppression was low. Enhanced responses were a prominent feature of this group. Prolongation of *M. leprae* contact to 6 days (which was the optimum time of antigenic stimulation) increased the per cent suppression in some untreated lepromatous patients. Interestingly two of the patients showing maximal suppression in this group had received treatment and showed higher baseline Con A responses than the others. (iii) Healthy contacts who were known to respond to *M. leprae* antigens in the lymphocyte transformation test showed a third pattern. Suppression in this group occurred late (i.e. day 6) and replaced the enhanced responses noted in the 4-day period, thereby showing *in vitro* antigenic influences which were half-way between the two disease groups.

Thus *M. leprae*-induced *in vitro* suppression of mitogenic responses was more consistently observed in tuberculoid leprosy and showed different time-kinetics in the two disease groups. Enhanced Con A responses in the presence of *M. leprae* were observed in patients with lepromatous leprosy. Prolongation of *M. leprae* contact to 6 days induced uniform suppressor activity in healthy contacts, tuberculoid patients and some lepromatous individuals.

In vitro lymphocyte transformation to PPD was consistently suppressed by *M. leprae* antigens in all three groups. The percentage inhibition was similar in tuberculoid and lepromatous patients and was marginally lower in the healthy contacts. The degree of inhibition to PPD showed less variability and was greater than the inhibition noted in the mitogen responses.

The present results on antigen-induced suppressor activity in leprosy patients are similar to the Con A-inducible suppression noted in our earlier studies (Nath *et al.*, 1979). In both studies, tuberculoid patients showed increased inducible suppressor activity whereas the lepromatous patients had lower suppression or enhancement of *in vitro* lymphocyte transformation. Moreover, the latter group was observed to have reduced numbers of T cells bearing Fc receptor for IgG (Singh & Nath, 1980).

The results on day-6 cultures in our study are similar to the observations of Bjune (1979) who also did not find differences in the patient groups in the antigen-induced suppression of sub-optimal PHA responses. As suggested by Bjune (1979), it is possible that the stimulation noted in some of our lepromatous patients may be due to the type of antigen used. Autoclaved *M. leprae* bacilli were used as antigen on the assumption that both cytoplasmic and surface antigens would be exposed due to non-integrity of cell membranes in dead bacilli.

Our findings on day-4 Con A responses are in conflict with the observations of Mehra *et al.* (1979) who adopted a similar *in vitro* system (day 3) and assayed suppressor effects using Dharmendra antigen. The patients included in their study had been treated whereas most of our patients and those of Bjune (1979) were untreated.

The different pattern of time-kinetics and the amount of suppression induced in each of the disease groups draws attention to the possibility of different immunoregulatory suppressor

mechanisms being operative in tuberculoid and lepromatous forms of leprosy. Further, it would appear from the *in vitro* studies that both general (Nath *et al.*, 1979; Singh & Nath, 1980) and antigen-related (Bjune, 1979; Mehra *et al.*, 1979) suppression may exist in leprosy.

Our earlier data had indicated that in the high-resistant form of leprosy, a subset of T cells may be responsible for suppression (Nath *et al.*, 1979). The present study lends support to the fact that antigen-induced *in vitro* suppression also correlates with the optimal immune responses and elimination of the causative organism observed in tuberculoid leprosy. On the other hand, the loss of suppressor activity in lepromatous leprosy may be reflected in loss of negative feedback mechanisms controlling a favourable immune response and unwanted antibody production.

It is possible that different cell types bring about suppression at different stages of the disease as is indicated in murine leprosy (Bullock *et al.*, 1979). Some workers have suggested that adherent cells can inhibit cellular responses in lepromatous leprosy (Hirschberg, 1978; Mehra *et al.*, 1979). However, the data on the role of macrophages in human leprosy are difficult to interpret, in view of the fact that mixed lymphocyte macrophage responses have involved allogeneic mixtures (Hirschberg, 1978; Mehra *et al.*, 1979).

Immunoregulation would be the result of a dynamic equilibrium between suppressor and helper cells. Genetic factors (Stoner, 1979), quantum of antigenic load and types of antigenic determinants released in different stages of the disease may influence this equilibrium. In addition, general immunosuppressive effects may be generated which would alter the final outcome of the disease. Interestingly in the borderline form of leprosy where cell-mediated immunity is relatively poor, periodic and natural enhancement of cellular responses are observed. These appear to correlate with neural and dermal inflammation (Barnetson *et al.*, 1975; Bjune *et al.*, 1976) and may reflect changes in equilibrium due to released *M. leprae* antigens. The factors influencing favourable immunoregulation to *M. leprae* antigens require attention for understanding the pathogenesis of leprosy and for the planning of immunoprophylactic measures.

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