Clin. exp. Immunol. (1981) **44**, 491–500.

Studies on T cell subsets and functions in leprosy

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(Accepted for publication 19 December 1980)

SUMMARY

T cell subsets and T cell functions were explored in 31 leprosy patients with the following methods: determination of the percentages of the different T cell subpopulations defined by monoclonal antibodies directed at total T cells, helper T cells and suppressor/cytotoxic T cells; measurement of the *in vitro* proliferative responses to mitogens; study of the concanavalin A-induced suppressive activity, assessed on MLC; measurement of delayed-type hypersensitivity by skin testing. The confrontation between immunological lepromatous patients without type-2 reaction (erythema nodosum leprosum), (2) lepromatous patients without ENL (erythema nodosum leprosum), (2) lepromatous patients with recent ENL and (3) tuberculoid patients. Unexpectedly, groups 1 and 3, although differing strongly in their clinical status and their sensitivity to lepromin (absent in group 1 and strong in group 3), showed a similar immunological profile with a normal percentage of T cells and a normal distribution of T cells among the major T cell subset contrasting with a moderate decrease of proliferative responses to mitogens and impaired delayed-type hypersensitivity reactions. Concanavalin A-induced suppressive activity was type-2 reaction) strongly differed from both other groups, showing striking abnormalities other groups, showing striking abnormalities of the repartition of the T cell subsets, with increased percentages of helper T cells and decreased percentages of suppressor T cells, and elevated proliferative responses to mitogens. Concanavalin A-induced suppressive activity was reduced in most patients of this group. It is suggested that this imbalance between T cell subsets contributes to the occurrence of ENL reactions in lepromatous patients.

INTRODUCTION

Leprosy is an infectious disease caused by *Mycobacterium leprae* usually affecting only a few individuals among an exposed population. The extent of the bacillary invasion and the clinical symptoms of the disease depend on the ability of the exposed individuals to mount an efficient cell-mediated immune response against *M. leprae* (Turk, 1976). The widely accepted clinical histopathological classification of leprosy into five groups (Ridley & Jopling, 1966) defines various grades of resistance to *M. leprae*. On the lepromatous side of this spectrum patients exhibit a

Abbreviations: M. leprae = *Mycobacterium leprae*, ENL = erythema nodosum leprosum, PBL = peripheral blood lymphocytes, FITC-GAM = fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody, SRBC = sheep red blood cells, PBMC = peripheral blood mononuclear cells, FCS = fetal calf serum, SIg = surface immunoglobulin, PHA = phytohaemagglutinin, Con A = concanavalin A, PWM = pokeweed mitogen, MLC = mixed leucocyte culture, DTH = delayed-type hypersensitivity, DDS = 4:4′diamino diphenyl sulphone.

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deficiency of T cell-associated responses to *M. leprae* and also some degree of non-specific depression of cell-mediated immunity (Godal, 1978). Some lepromatous patients are prone to a particular reactional state, erythema nodosum leprosum (ENL), which is thought to be an immune complex disease, having features in common with Arthus' phenomenon (Wemambu *et al.*, 1969).

Patients on the tuberculoid side of the spectrum show a T cell-mediated responsiveness to *M. leprae* antigens, as demonstrated by the lepromin test. They do not present ENL reactional states.

The mechanism of T cell deficiency in lepromatous leprosy remains obscure. Several explanations have been proposed: specific clonal deletion, antibody-mediated suppression of cell-mediated immunity, and, more recently, active suppression by suppressor cells and/or macrophages (Bullock, Carlson & Gershon, 1978; Mehra *et al.*, 1979; Turcotte, 1978).

Effective immunity against infectious agents results from an adequate balance between the different components of the regulation system of the immune responses. Since T cells play a central role in these regulatory mechanisms, alterations of immunity as seen in leprosy could conceivably be associated with abnormalities of the regulatory T cell subsets.

Monoclonal antibodies directed against specific human T cell subsets provide a new tool for the analysis of T cell subpopulations in man (Reinherz & Schlossman, 1980). In the present work, we have simultaneously studied in leprous patients T cell subsets defined by their surface markers, and T cell functions both *in vitro* and *in vivo*. The data reported here demonstrate that, whereas tuberculoid and non-reactional lepromatous patients show a non-specific moderate decrease of T cell immune responses, without concomitant alterations of T cell markers, lepromatous patients suffering ENL episodes have a completely different immunological profile, with decreased suppressor T cell number and activity and a simultaneous increase in helper T cell number and proliferative responses to mitogens.

MATERIALS AND METHODS

Patients and controls. Thirty-one leprosy patients originating from the West Indies (14), Africa (eight), South East Asia (eight) and Spain (one) were examined at the Hôpital Saint Louis, Pavillon de Malte, Paris. Patients were divided into three groups (Table 1). Group 1 was composed of nine polar lepromatous (LL) or borderline lepromatous (BL) patients with no recent history of ENL. Group 2 included nine other LL or BL patients who had suffered from ENL less than 2 months prior to the investigation. Group 3 consisted of 13 borderline tuberculoid (BT) or polar tuberculoid (TT) patients.

Some patients had been examined by us prior to treatment and had been classified at that time according to the Ridley–Jopling scale (four in group 1, five in group 2 and seven in group 3). For other patients, who were first seen when already treated, classification was based upon clinical and histological data obtained at that time. Three patients initially classified as BL patients had experienced reversal reactions 1 year before the present investigation and have been finally classified as BT patients. Only five out of 13 tuberculoid patients kept active granuloma sites when studied.

The diagnosis of ENL was established according to the usual criteria: sudden eruption of painful erythematous nodules, with dermal oedema and polymorph infiltration, and occasional vasculitis. Fever and leucocytosis were present in almost all cases. Neuritis, iritis, orchitis and arthritis were present in a few cases. No patient in groups 1 or 2 had had a recent reversal reaction.

Controls were healthy volunteers, staff members of our department or blood bank donors, most of them Caucasians, 20 to 60 years old, with approximately the same proportion of males and females.

Skin tests. An equal amount (0·1 ml) of each of the following antigens was injected i.c. into the outer part of the arm: purified tuberculin, 10 units, and candidin diluted 10^{-3} (Institut Pasteur, Paris, France); varidase (50 units streptokinase, 12·5 units streptodornase; Lederle, Madrid, Spain); streptococci (a suspension of 8×10^7 autoclaved bacilli from groups A, B, C, D, N and O, Institut Mérieux, Lyon, France). Tests were read after 48 hr and recorded as follows: no clinical sign = negative; erythema and faint swelling = $\pm$; papular induration of less than 5 mm

Table 1. General characteristics of the patients

Patients	Age (years)	Classification	Number of treated patients (duration of therapy)*	Number of bacillary patients (bacterial index)†
Group 1				
9 lepromatous patients without recent ENL	22–78	7 LL, 2 BL	8 (2–33 years)	3 (1+–5+)
Group 2				
9 lepromatous patients with recent ENL	21–55	7 LL, 2 BL	8 (3–29 years)	8 (2+–4+)
Group 3				
13 tuberculoid patients	18–64	9 TT, 4 BT	11 (1 month–26 years)	2 (1+)

* In group 1, four patients received rifampicin, alone or in association with sulphamide (plus clofazimine in two cases); four patients received DDS and/or sulphamide. In group 2, five patients received rifampicin, alone or in association with DDS or sulphamide (plus clofazimine in one case and thalidomide in two cases); three patients received DDS or sulphamide (plus clofazimine in one case and thalidomide in one case). In group 3, one patient received rifampicin alone, four patients received DDS alone, and six received sulphamide and/or clofazimine. Duration of disease was similar to duration of therapy except in one case.

† Bacterial index was recorded as described by Ridley & Hilson (1967) as the average count of three to five smears.

diameter = 1+; 5 to 12 mm = 2+; 12 to 20 mm = 3+; more than 20 mm = 4+. All the tests were read by the same examiner.

For each individual the total number of + was recorded and used as an index for DTH level.

Monoclonal antibodies. Monoclonal antibodies directed against various human T cell antigens produced as already described (Reinherz *et al.*, 1979a, 1979b) by mouse hybridomas, obtained from cell fusion between mouse myeloma cells and normal splenocytes from mice immunized with human thymocytes or E rosetting human peripheral blood lymphocytes (PBL), were kindly donated by P. C. Kung and G. Goldstein, Raritan, New Jersey, USA. Previous studies demonstrated that some of these antibodies recognized all peripheral T cells among human PBL, whereas others reacted with different PBL T cell subsets committed to different functional programmes (Reinherz & Schlossman, 1980).

In this study, we have used three of these antibodies: OKT3, reacting with all peripheral T cells; OKT4, recognizing only peripheral T cells with helper inducer function and OKT8, directed against a T cell subset expressing both suppressor function and cytotoxic activity (Reinherz *et al.*, 1980a, 1980b).

Fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (FITC-GAM). Goat anti-mouse IgG antiserum (Miles Laboratories, Elkhart, USA) was first passed through a Sepharose 4B-CN column coated with human IgG in order to eliminate possible antibodies cross-reacting with human IgG determinants. Then, anti-mouse IgG antibodies were purified on a Sepharose 4B-CN column coated with mouse IgG. Anti-mouse IgG antibodies at a concentration of 10 mg/ml were then conjugated to fluorescein isothiocyanate according to the technique previously described (Winchester *et al.*, 1975).

Enumeration of T cell subsets with monoclonal antibodies. Peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll–sodium metrizoate gradient (Telebrix, Paris, France). Forty-five microlitres of a 10–20 × 10^6/ml PBMC suspension in Hanks' medium containing 0·2% sodium azide

and 5% fetal calf serum (FCS) (Eurobio, Paris, France) were mixed with 5 μl of the appropriate dilution of each antibody (OKT3: 1/5; OKT4: 1/20; OKT8: 1/25) in haemolysis tubes and left for 30 min at 4°C in an ice bath. Cells were then washed twice and the pellet was added with the same medium containing FITC-GAM (1/50 of the stock solution). Cells were left again for 30 min in an ice bath at 4°C, then washed twice. The pellet was resuspended in 50 μl medium and the percentage of fluorescein-labelled cells was scored immediately under a fluorescent microscope.

Proliferative responses to mitogens. Proliferative responses to different mitogens—PHA (DIFCO, Detroit, USA), 2 μg/ml, Con A (Pharmacia, Uppsala, Sweden), 50 μg/ml, and pokeweed mitogen (PWM) (GIBCO, Grand Island, USA), 1/20 of the stock solution—were studied as described elsewhere (Charpentier, Carnaud & Bach, 1979) by measuring ^{3}H-thymidine incorporation. The mean ^{3}H-thymidine incorporation of quadruplicates was calculated for each mitogen, and from this was subtracted that obtained without mitogen. Results are expressed for each patient's values as a percentage of the c.p.m. obtained with the PBMC of the normal subject tested on the same day, since overall levels of proliferative responses varied somewhat from one experiment to another.

Con A-induced suppressive activity. The suppressive activity of Con A-activated PBMC was assessed on MLC as already described by Sakane & Green (1977) with slight modifications allowing the test to be performed with a single blood sample. In brief, PBMC suspended at 10^6/ml in RPMI containing 10% FCS, 200 units/ml penicillin and 200 μg/ml streptomycin were incubated with Con A (Pharmacia, 7·5 μg/ml) in Falcon flasks (25 cm^2) for 48 hr at 37°C in a humidified CO_2 incubator. Control cells were incubated in the same conditions without Con A. Forty-eight hours later, 10^5 Con A-stimulated cells or uneducated control cells were added, after mitomycin C treatment, to a mixed leucocyte culture set up in Falcon II microplates with 10^5 autologous responder cells that had been stored separately at 37°C in RPMI containing 20% human AB serum, and 10^5 mitomycin-treated allogeneic cells. Five days later, ^{3}H-thymidine incorporation in MLC was measured as described elsewhere (Charpentier *et al.*, 1979). The suppressive activity on MLC of Con A-stimulated cells was expressed as follows:

$$100 \times \frac{\text{c.p.m. in MLC with control cells} - \text{c.p.m. in MLC with Con A-activated cells}}{\text{c.p.m. in MLC with control cells}}.$$

Statistical methods. Student's *t*-test was used for all statistical analysis.

RESULTS

Within each group of patients defined as stated above (lepromatous without ENL, lepromatous with ENL and tuberculoids), data obtained from BL or LL patients on one hand, and from BT or TT patients on the other hand, did not differ and have therefore been pooled.

T cell subsets defined by monoclonal antibodies (Table 2)

The percentage of OKT3$^+$ cells was normal in both groups of lepromatous patients (with or without ENL) and slightly decreased in tuberculoid patients. However, the balance between the two major T cell subsets, OKT4$^+$ and OKT8$^+$, was strongly altered in lepromatous patients with ENL, with a decreased percentage of the OKT8$^+$ suppressor T cell subset and an increased percentage of the OKT4$^+$ helper T cells. These changes appeared still more clearly when OKT4$^+$ and OKT8$^+$ cell numbers were expressed for each patient as the percentages of the total number of T cells (i.e. the percentage of OKT3$^+$ cells) since such calculation eliminates the fluctuation of OKT4$^+$ and OKT8$^+$ T cell percentages due to variable content of PBMC in monocytes. Thus, the ratio of OKT4$^+$/OKT8$^+$ T cells, which expressed at best the balance between the two major T cell subsets in peripheral blood, was elevated among the lepromatous patients suffering ENL episodes, as compared to the two other groups of patients and to the normal subjects.

In both other groups of patients (1 and 3) the percentages of OKT8$^+$ among PBMC or among peripheral blood T cells, and the ratios of OKT4$^+$/OKT8$^+$ T cells were not different from those of normal subjects.

Table 2. T cell subsets in leprosy patients analysed by indirect immunofluorescence with anti-human T cell monoclonal antibodies

Patient groups	Antibodies					
	Per cent positive cells among peripheral blood mononuclear cells*			Per cent positive cells among T cells ($OKT3^+$)*		Ratio
	OKT3	OKT4	OKT8	OKT4	OKT8	% $OKT4^+$/% $OKT8^+$*
1. Lepromatous without ENL	64·6±4·1 (9)	40·1±3·2 (9)	25·0±1·8 (9)	62·3±4·1	39·4±3·3	1·64±0·17 (9)
2. Lepromatous with ENL	61·4±4·2 (9)	43·9±3·2 (8)	17·5±2·0† (9)	75·4±7·4‡ (8)	28·6±2·6§ (9)	2·91±0·37¶ (8)
3. Tuberculoid	56·3±3·8** (13)	38·1±3·9 (12)	21·7±1·8 (13)	69·9±5·5 (12)	39·1±3·0 (13)	1·86±0·20 (12)
4. Normal subjects	66·7±1·7 (41)	41·4±1·4 (41)	25·7±1·3 (41)	63·0±2·2 (41)	38·7±1·8 (41)	1·80±0·13 (41)

* Mean ± s.e. (number of cases is given in parentheses).
† Significantly different from group 1 value ($P<0·02$) and group 4 value ($P<0·01$).
‡ Significantly different from group 4 value ($P<0·05$).
§ Significantly different from groups 1 and 3 values ($P<0·05$) and from group 4 values ($P<0·02$).
¶ Significantly different from group 1 value ($P<0·01$), group 3 value ($P<0·02$) and group 4 value ($P<0·001$).
** Significantly different from group 4 value ($P<0·01$).

Table 3. Proliferative responses to mitogens in leprosy patients

Patients	Mitogens (% of the response of normal controls)*		
	PHA	Con A	PWM
1. Lepromatous without ENL	62·7 ± 13·4† (9)	66 ± 17·4 (9)	65·9 ± 16·6 (9)
2. Lepromatous with ENL	132·7 ± 12·9‡ (9)	136 ± 20·0§ (9)	191·7 ± 28·8‡ (9)
3. Tuberculoid	80·4 ± 9·0 (13)	78·2 ± 8·5 (13)	78·3 ± 16·3 (13)

* Mean ± s.e. of c.p.m. obtained from 14 normal control cultures; with PHA: 61,867 ± 4,908; with Con A: 40,664 ± 4,508; with PWM: 15,221 ± 279; without mitogen: 921 ± 82.

† Mean ± s.e.

‡ Significantly different from groups 1 and 3 values ($P<0·01$).

§ Significantly different from groups 1 and 3 values ($P<0·02$, $P<0·01$).

Proliferative responses to mitogens (Table 3)

Lepromatous patients without ENL reaction and tuberculoid patients behave similarly with regard to their proliferative responses to mitogens, which tend to be decreased (the response to PHA being the most consistently altered). On the other hand, lepromatous patients with ENL reaction showed much higher proliferative responses to mitogens than both other groups. Proliferation to PHA and PWM even exceeded that of normal controls ($P<0·05$ and $P<0·02$).

As shown in Fig. 1, the proliferative responses of leprosy patients to PHA, Con A and PWM, expressed as percentages of those of normal controls, were found to be significantly correlated with the ratio of $OKT4^+$ (helper cells)/$OKT8^+$ (suppressor cells) ($P<0·02$, $P<0·01$ and $P<0·01$ respectively).

Con A-induced suppressive activity (Fig. 2)

For technical reasons fewer patients could be tested for the ability of their PBMC to develop suppressive activity after Con A stimulation. Four out of six lepromatous patients suffering ENL showed a drastic decrease of the suppressive index, whereas none of the other patients—either lepromatous without ENL or tuberculoid—had a diminished Con A-induced suppressive activity.

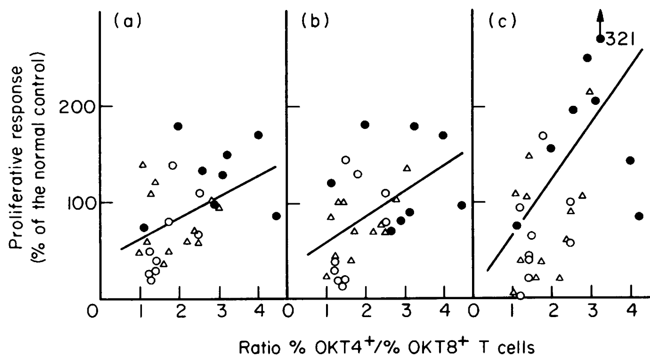

Fig. 1. Correlation between proliferative responses to mitogens and changes in T cell subsets defined by monoclonal antibodies in leprous patients. For each individual patient, proliferative responses to PHA (a), Con A (b) and pokeweed mitogen. (c) were plotted against the ratio % $OKT4^+$/% $OKT8^+$ T cells. (o) Lepromatous patients without recent ENL reaction; (•) lepromatous patients with recent ENL reaction; (△) tuberculoid patients. The regression curves were calculated by the *least square* method.

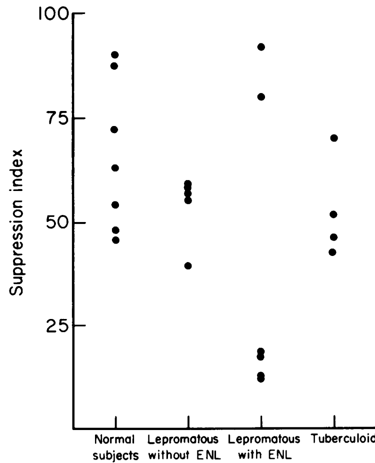

Fig. 2. Con A-induced suppressive activity in leprous patients. Con A-stimulated PBMC from leprous patients or normal subjects, or control cells cultured without Con A, were added to an MLC using autologous responder cells. Suppression index of the MLC response was calculated as follows:

$$I = 100 \times \frac{\text{c.p.m. in MLC plus control cells} - \text{c.p.m. in MLC plus Con A-stimulated cells}}{\text{c.p.m. in MLC plus control cells}}.$$

Intradermal skin test

Delayed-type hypersensitivity (DTH) to four different antigens was evaluated in leprous patients and normal subjects by intradermal skin tests. DTH reactions were found to be decreased in all groups of leprous patients (mean DTH index $\pm$ s.e. in group 1: $6{\cdot}42 \pm 0{\cdot}71$; in group 2: $4{\cdot}5 \pm 0{\cdot}96$; in group 3: $6{\cdot}27 \pm 1{\cdot}02$; in normal controls: $9{\cdot}46 \pm 0{\cdot}44$). No significant differences appeared between these three groups of patients.

DISCUSSION

We have simultaneously explored in leprous patients and in normal controls T cell markers, proliferative responses to mitogens, Con A-induced suppressor T cell activity and *in vivo* DTH reactions to several antigens. Confronting the results of these investigations and the clinical data, we could delineate three main groups of patients: lepromatous patients (LL or BL) without ENL, lepromatous patients (LL or BL) with recent ENL reaction and tuberculoid patients (TT or BT).

Lepromatous patients without ENL show depressed proliferative responses to PHA, Con A and PWM, impaired DTH reaction to PPD, varidase, streptococci and candidin, as already described by others (Bulloch & Fasal, 1971; Nelson *et al.*, 1971; Nath *et al.*, 1977), contrasting with normal T cell percentages in peripheral blood and normal T cell distribution between OKT4$^+$ helper and OKT8$^+$ suppressor T cells. Con A-induced suppressor T cell activity was not altered. As shown by Reinherz *et al.* (1979b), the proliferative response to PHA is mediated by OKT4$^+$ T cells and to a lower degree by OKT8$^+$ T cells, whereas both subpopulations respond equally well to Con A. PWM-induced proliferation, although involving mainly B cells, requires T cell help, and is controlled by the balance between OKT4$^+$ and OKT8$^+$ T cells. DTH reactions, explored *in vitro* by lymphocyte transformation studies in the presence of soluble antigens, are only mediated by OKT4$^+$ T cells (Reinherz *et al.*, 1979b). Thus one would have expected an absolute or relative decrease of the number of OKT4$^+$ T cells and a normal or relatively increased number of OKT8$^+$ T cells. This apparent contradiction between marker and function studies may have several explanations. First, a serum suppressor factor, as already described by other authors (Nelson *et al.*, 1975; Bjune & Barnetson, 1976), could depress lymphocyte proliferation: this is unlikely since all *in vitro* studies were performed in FCS-containing medium, without autologous serum, unless it is assumed that such factors could have irreversible effects on lymphocytes. Second, macrophages or a few clones of suppressor T cells could be activated specifically by antigens of *M. leprae in vivo* and

non-specifically suppress other T cell responses, as suggested by several authors (Bjune, 1978; Mehra *et al.*, 1979).

Lepromatous patients with recent ENL reaction behave quite differently from the preceding group since they show elevated responses to mitogens, often above those of normal controls, imbalance between helper and suppressor T cells in favour of the former, and in some, but not all cases, a drastic decrease of the Con A-induced suppressor T cell activity. Our ENL patients showed high mitogen responsiveness despite the fact that most of them had a heavy bacterial load (see Table 1), a circumstance previously reported to be associated with low responses to PHA in lepromatous patients (Nath *et al.*, 1977). Taken together, these data support the hypothesis that a failure of $OKT8^+$ suppressor T cells, assessed by both marker and function studies, is associated with a simultaneous increase of $OKT4^+$ T cell numbers and functions. DTH reactions to recall antigens, although mediated by $OKT4^+$ T cells, are not increased, suggesting that primary sensitization with these antigens failed to elicit specific memory cells in these formerly immunodepressed patients. Thus although they have partly recovered T cell-mediated immunity, they still lack the memory cells necessary to mount recall responses.

Our findings raise the question of the possible role of this imbalance between helper and suppressor T cells in the occurrence of ENL episodes. ENL reactions have been attributed to the deposition of circulating immune complexes (Rojas-Espinosa, Mendez-Navarrete & Estrada-Parras, 1972) and/or to their local formation in tissues (Wemambu *et al.*, 1969; Bjorvatn *et al.*, 1976). Since lepromatous patients produce excessive amounts of antibody against *M. leprae*, abundant in their tissues, but also against several autoantigens (Turk & Bryceson, 1971), a transient failure of suppressor T cell activity may lead to an increased IgG production and to the subsequent formation of immune complexes as described during the acute phases of SLE (Sakane, Steinberg & Green, 1978).

Our data are in agreement with several other reports showing increased responsiveness to PHA (Lim *et al.*, 1975; Anders, MacAdam & Anders, 1977) in lepromatous patients presenting with ENL reactions. Similarly, Waldorf *et al.* (1966) and Rea & Levan (1980) found increased susceptibility to DNCB sensitization in lepromatous patients with ENL as compared to other lepromatous patients. Our data contradict those of Nath *et al.* (1979) who reported a depressed suppressive activity of Con A-stimulated cells on mitogen-induced proliferation in lepromatous patients without ENL and a normal suppressive activity in lepromatous patients with ENL. This apparent contradiction might be explained by the different assay we used for assessing the suppressive activity of Con A-stimulated cells, and would suggest that the proliferative response in MLC and mitogen-induced proliferation would be regulated by different mechanisms. Alternatively, differences in recruitment of patients (most of our patients were already treated in contrast to those of Nath *et al.*) may explain these opposite results.

These findings also pose the problem of whether lepromatous patients with ENL reaction show a permanently different immune status than other lepromatous patients, making them more susceptible to the occurrence of such reactions, or whether such modification of immune status and subsequent ENL reactions may occur spontaneously—and transiently—in any lepromatous patient. This important problem may be solved only by longitudinal studies of the same patients over long periods of time.

Tuberculoid patients show a moderate decrease of their proliferative responses to mitogens, a more pronounced decrease of DTH reaction, and a normal balance between $OKT4^+$ and $OKT8^+$ T cells. Thus immunological data obtained from these patients are quite similar to those observed in our group of treated lepromatous patients without ENL. Although it is generally accepted that tuberculoid leprosy induces a lesser degree of non-specific immunosuppression than lepromatous leprosy (Godal, 1978), some authors have already reported a moderate depression of cell-mediated immunity in tuberculoid patients (Bullock, 1968; Han, Weiser & Kau, 1971), which raises the question of a pre-existing immunodeficiency state in a subfraction of the population of subjects exposed to *M. leprae*, allowing them to develop clinical infection.

Prospective studies on T cell subsets analysed by both surface markers and functional *in vitro* assays in populations chronically exposed to *M. leprae* should provide better insight on the pathophysiological mechanism of specific and non-specific immunodeficiency in leprosy.

We wish to thank Dr B. Flageul, Dr J. Pennec and Dr M. Taieb for their help in collecting pertinent clinical data, and Mr G. Dumas and Mrs J. Maciazek for their skilful technical assistance.

This work was supported in part by a grant from the Association des Oeuvres Hospitalières Françaises de l'Ordre de Malte.

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