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Selective loss of *Mycobacterium leprae* responsiveness of circulating lymphocytes in primary neuritic leprosy

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Summary Sixteen patients with Primary neuritic leprosy characterized by neural involvement in the absence of dermal lesions were studied. Eight healthy contacts from non-endemic areas formed the control group. T and B cell numbers as well as lymphocyte transformation responses to Concanavalin A (Con A), Phytohaemagglutin (PHA) and PPD were not found to be significantly decreased in the majority of the neuritic patients. However, there was a selective uniform lack of *M. leprae* induced lymphocyte transformation in this group (Median stimulation index – 1.05). In 3 of the patients, the antigen specific unresponsiveness was present even after 5 years of chemotherapy. The clinical extent of neural involvement and serum factors did not appear to contribute to the antigen specific deficit observed in this form of leprosy.

Introduction

The clinical features of leprosy are predominantly associated with skin and nerve involvement, the causative organism, *Mycobacterium leprae*, having a particular affinity for skin and neural tissue. Being an obligate intracellular organism it mainly resides in the tissue macrophages and Schwann cells. Dermal nerve involvement is observed in the tuberculoid, borderline and lepromatous forms of leprosy, (Ridley & Jopling, 1966).[1] The larger peripheral nerves appear to be affected early in tuberculoid leprosy and only in the later phases of lepromatous leprosy (Cochrane, 1964;[2] Dharmendra, 1978).[3] In borderline leprosy, neuritis during reactions, forms a serious complication which may lead to permanent damage to the nerve (Barnetson *et al*, 1978).[4]

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Nerves may also be affected primarily in leprosy without an apparent involvement of skin or dermal nerves. Controversy exists as to the exact position of this form of leprosy in the general spectrum of the disease. Neural anaesthetic lesions were recognized as a separate entity in the Cairo Congress Classification (1938)[5] and were grouped into lepromatous, tuberculoid and indeterminate forms in the Madrid Congress Classification (1953).[6] The latter classification was mainly based on the number of nerves involved in the absence of skin lesions. Pure neuritic patients are frequently seen in India, though the occurrence of this form of leprosy has regional variations (Noordeen, 1972).[7] Some workers classify such lesions in a manner similar to dermal lesions. However, according to the Indian system of classification, primary or pure neuritic leprosy is grouped as a separate clinical entity (Dharmendra & Chatterji, 1953).[8] Neuritis in nerves draining active or healed lesions has been designated as secondary neuritic leprosy. The primary neuritic group is further subdivided into mononeuritic (single nerve involvement) or polyneuritic (two or more nerves affected) leprosy (Dharmendra, 1978).[3] The patients included in the current study have been graded according to the above criteria.

Strong evidence exists to indicate that host-determined immunological responses are responsible for the leprosy spectrum (Ridley & Jopling, 1966;[1] Myrvang *et al*, 1973).[9] Yet the mechanisms leading to nerve damage in leprosy remain unknown. Recent reports indicate that patients undergoing reactions in borderline leprosy develop neuritis which is associated with enhanced lymphocyte transformation to *M. leprae* antigens (Barnetson, 1978).[4] As far as we are aware, immunological data in patients with primary neuritic leprosy is scant. The present study was therefore undertaken on 16 patients with primary polyneuritic and mononeuritic forms of leprosy. Enumeration of (a) circulating T and B cells and (b) lymphocyte transformation to T cell mitogens, specific *M. leprae* antigen and PPD were undertaken to assess the immunological status of these patients.

Materials and methods

SUBJECTS

Sixteen patients between the age groups of 23 to 70 years attending the leprosy clinics of All India Institute of Medical Sciences and Lok Nayak Jaya Prakash Narayan Hospital, Delhi, were included in this study. Fourteen were male and 2 were females. None of the patients had active or healed dermal lesions. Four patients had mononeuritic leprosy, 3 had ulnar nerve involvement and one had affection of the lateral popliteal nerve; 12 others had thickening of 2 or more nerves and were placed in the polyneuritic group (Dharmendra, 1978).[3] One patient with mononeuritic and 2 with polyneuritic leprosy had been treated with DDS (300 mg/week) for 5 years. Detailed neurological examination for

sensory and motor functions were performed on all the patients (Table 1). Lepromin skin tests using whole autoclaved bacilli were available in 8 patients. The other patients were not obtainable for reading of the skin test at the appropriate time. Eight healthy individuals from the laboratory and clinical staff between the age groups of 25 to 50 years, who were not residents of endemic areas but had been constantly exposed to leprosy patients and *M. leprae* antigens for 3–5 years were included as a control group of healthy contacts.

Table 1. Clinical status of 16 patients with primary neuritic leprosy

	Number of patients	
	Polyneuritic leprosy	Mononeuritic leprosy
Number of involved nerves		
(a) One	Nil	4
(b) Two to four	12	Nil
Nerves involved		
(a) Ulnar	12	3
(b) Median	10	Nil
(c) Radial	2	Nil
(d) Lateral popiteal	5	1
(e) Posterior tibial	3	Nil
Muscle changes		
(a) Flattening only	2	Nil
(b) Wasting	12	4
(c) Deformity	5	1
Sensory loss		
(a) Thermal	12	4
(b) Touch	12	4
Trophic changes		
(a) Dry glossy skin	10	2
(b) Blisters	Nil	Nil
Motor nerve conduction velocity		
(a) Normal	4	Nil
(b) Delayed	8	4

NERVE CONDUCTION STUDIES

A medelec 2 channel EMG machine (UK) was used to study the affected nerves in the patients. Motor nerve conduction velocities below 40 m/sec were considered abnormal (The normal Indian subjects showed a range of 40–60 m/sec).

NERVE BIOPSIES

Biopsies were only performed on the radial cutaneous nerves in 4 patients with multiple nerve involvement and sensory loss in the area supplied by the nerve. Biopsies were not attempted in patients with single nerve or only motor nerve involvement for ethical reasons. Histopathological specimens were stained with Haematoxylin and Eosin, and Ziehl Neelson Laxol. Fast blue followed by Periodic acid Schiff reagent was used for delineation of myelin.

SEPARATION OF LYMPHOCYTES

Lymphocytes were isolated from sterile heparinized venous blood (10 IU of preservative free Heparin per ml) by Ficoll-Isopaque density gradient centrifugation according to Boyum (1968).[10] The lymphocytes collected from the interface were contaminated with 5 to 15% monocytes and 1–2% neutrophils. They were washed twice with Eagles' Minimum Essential Medium and resuspended in RPMI 1640 (Gibco Biocult) to a concentration of 1×10^6 per ml. 10% pooled AB serum was added to the aliquots of cells used for lymphocyte transformation tests.

ENUMERATION OF T AND B CELLS

(i) T-cells were identified by spontaneous rosette formation with sheep red blood cells treated with AET (2 aminoethylisothiouronium bromide hydrobromide, Sigma Chemical Co) by a modified method of Kaplan and Clark (1974).[11]

(ii) B cells were identified by surface markers for immunoglobulin and activated complement as described earlier (Nath *et al*, 1977).[12]

(a) Immunoglobulin marker was identified by direct immunofluorescence using FITC conjugated antihuman immunoglobulin (Capell Labs). In brief, 3×10^6 lymphocytes in 100 μl of MEM were mixed with 50 μl of an optimum dilution of the fluorescein-labelled antiserum, incubated at 4°C for 45 min, washed thrice with MEM and finally suspended to 100 μl. One drop of the cell suspension was put on a slide covered with a coverslip and sealed with nail varnish. Fluorescence was visualized using a Carl Zeiss universal microscope with an HBO 200 mercury lamp and KP 500 excitation filter.

(b) Rosette formation with sheep erythrocytes coated with anti-sheep erythrocyte antibody and mouse complement was used to identify B cells with receptors for activated complement (Bianco Patrick & Nussensweig, 1970).[13] 300 lymphocytes were counted for each individual test.

LYMPHOCYTE TRANSFORMATION TEST

Microcultures were performed in round bottom microtitre plastic plates (Nunc Intermed, Denmark). Each well contained 2×10^5 lymphocytes in 200 μl of

RPMI 1640 buffered with Hepes (Gibco Biocult) and supplemented with 10% pooled AB serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cultures were incubated at 37°C in a humidified chamber containing 5% CO_2 in air. 25 μl of stimulants were added to test cultures and consisted of (i) Concanavalin A (Pharmacia Uppsala 600 μg/ml) and phytohaemagglutinin (Difco Labs 800 μg/ml); (ii) antigens: (a) PPD (Ministry of Agriculture and Fisheries, UK, 50 μg/ml). (b) Whole *M. leprae* were derived from skin nodules of untreated lepromatous leprosy patients by homogenization of skin and two washings with PBS. The bacilli were autoclaved prior to use. The concentrations used were 5×10^6 and 10^7 bacilli per ml: these were chosen after performing dose response curves on lymphocytes from normal subjects and tuberculoid patients. Mitogen and antigen stimulated cultures were terminated after 3 and 6 days respectively. Fourteen–sixteen hours prior to harvesting 25 μl of ^{3}H-thymidine containing 1 μCi (Bhabha Atomic Research, Trombay, Sp activity 6.4 mCi per mmol) was added to each culture. The cells were collected on glass fibre paper using automatic harvester. ^{3}H-thymidine incorporation was determined and the results given as counts per minute (CPM) in a liquid scintillation counter. All cultures were performed in quadruplicate.

Due to the long period involved in the study, lymphocytes from each set of patients were tested in parallel with control subjects. As technical variations inherent in such protracted studies are inevitable, the results were expressed as stimulation indices rather than counts per minute. A stimulation index above 2 was considered to indicate stimulation with particular reference to antigen stimulated cultures.

STATISTICAL ANALYSIS

The results were analysed by nonparametric methods using Mann Whitney U Test (Siegel, 1956).[14]

Results

CLINICAL FEATURES

The 16 patients studied were in the adult age group and predominantly male. Their general condition and nutritional status was good. Skin tests using Mitsuda type lepromin were negative in all eight of the patients studied. The details of the neurological features of the primary neuritic patients in this study are given in Table 1. It was noted that absence of dermal lesions, presence of nerve thickening with tenderness only on deep pressure and sensory loss in the area of nerve supply were universal features. Motor nerve conduction velocities were delayed in the majority of the patients studied. Mononeuritic and polyneuritic groups could not be distinguished by any objective criteria (except on

the basis of the number of nerves involved). Therefore, all neuritic patients were grouped together for the other parameters.

HISTOPATHOLOGY OF RADIAL CUTANEOUS NERVES

For ethical reasons biopsies of nerve trunks were not done. Four patients with polyneuritic leprosy who had sensory loss in the skin areas supplied by the radial cutaneous nerve were biopsied. Histological examination indicated thickening with multilayered appearance of the perineurium in all the nerves. Mild to moderate infiltration with mononuclear cells and lymphocytes was observed. Acid fast bacilli were seen in the perineurium and in many of the Schwann cells. Patchy demyelination as well as moderate nerve fibre damage was noted.

CIRCULATING T AND B CELL NUMBERS

Table 2 shows the percentage of T and B cells in the peripheral blood of neuritic patients and healthy contacts. It may be observed that the percentages of B cells are within the normal range. The T cell numbers on the other hand appear marginally reduced in the patients as compared to the normal subjects. This was not found to be statistically significant.

Table 2. Circulating T and B Cell numbers in patients with primary neuritic leprosy and healthy contacts

	Mononuclear cells		
	T Cells	Ig(+) Cells	EAC Rosettes
Healthy contacts (8)			
Range	58 – 77	10 – 16.5	8.6 – 14.4
Median	71.2	11.8	13.0
Mean ± SD	68.8 ± 9.1	12.7 ± 2.7	12.4 ± 2.4
Neuritic patients (16)			
Range	54 – 73	8 – 10.5	8.1 – 14
Median	67.8	10.5	12.0
Mean ± SD	65.9 ± 5.8	11.2 ± 2.26	10.4 ± 1.9

Figures in parentheses indicate number of individuals studied.
SD = Standard deviation.

LYMPHOCYTE TRANSFORMATION

(i) *T Cell Mitogens*: Peripheral blood lymphocytes from patients and healthy contacts were stimulated in parallel experiments with optimal doses of PHA and Con A. No difference in responsiveness to mitogens was noted between the mononeuritic and polyneuritic groups. Eleven of the primary neuritic patients showed lower stimulation indices to Con A and 7 showed decreased levels to

PHA as compared to the control subjects (Fig. 1). The median values for Con A and PHA in the patient group were 29.0 and 43.9 respectively whereas the control group showed medians of 66.7 and 73.4 for Con A and PHA respectively. Statistical analysis by non-parametric methods (Mann Whitney U Test) did not reveal any significant differences between the neuritic and control groups. This may be due to the wide range of responsiveness to T cell mitogens seen in the patients.

(ii) *Antigens:* PPD and autoclaved integral *M. leprae* were used for *in vitro* antigen stimulation of lymphocytes. Fig. 1 shows the stimulation indices of individual patients and healthy contacts. It may be noted that responsiveness to PPD also showed a wide scatter and did not indicate any statistical difference between the two groups. The Median stimulation index in the neuritic and control groups was 4.7 and 7.4 respectively.

The important feature of this study was the specific lack of responsiveness to *M. leprae* in the neuritic group. Seven of the eight healthy contacts on stimulation showed *M. leprae* stimulation indices ranging from 2.0 to 3.8 with a Median value of 2.5. In contrast all the neuritic patients studied showed a stimulation index which ranged from 0.63 to 1.4 (Median 1.05). The statistical difference between the two groups was found to be highly significant ($p < 0.002$).

Discussion

Neural involvement is a predominant feature of leprosy in the presence and the absence of dermal lesions. *M. leprae* has been shown to reside mainly in the Schwann cells (Job, 1971;[15] Pearson & Ross; 1975)[16] and infrequently within the axons of peripheral nerves (Boddingius, 1974).[17] Most of the studies to date focus attention on the status of dermal and peripheral nerves in the general leprosy spectrum. Clinical (Cochrane, 1964;[2] Dharmendra, 1952;[18] 1978;[3] Pearson & Ross, 1975),[16] electrophysiological (Antia *et al*, 1975;[19] Mehta *et al*, 1975)[20] and extensive morphological evidence (Iyer, 1965;[21] Job & Desikan 1968;[22] Job, 1971;[15] Dastur, Rammohan & Shah, 1972;[23] 1973;[24] Dastur, 1978;[25] Pearson & Weddell, 1975)[26] regarding the involvement of nerves is available in the literature. However, the pure or primary neuritic form of leprosy has not been investigated fully and the consensus does not exist as to its exact status in the leprosy spectrum.

Immunological studies pertaining to nerve involvement in leprosy have been few. In recent years, Bjune *et al* (1976)[27] and Barnetson (1978)[4] drew attention to enhanced antigen induced lymphocyte responses in boderline leprosy patients who developed neuritis during reactions. Primary neuritic leprosy as seen in Indian patents is a unique example of the preferential predilection of *M. leprae* for the peripheral nerves rather than the dermal nerves of

skin (Dharmendra, 1978).[3] This feature is even more striking when it is realized that in the peripheral nerve as well as in the dermal nerves, the host cell for *M. leprae* is the Schwann cell. Though strong evidence exists to indicate that host determined immunological factors are responsible for the varied spectrum of leprosy, no data is available on the immunological status of this neuritic group of patients.

In the present study, except for the number of nerves affected, differences regarding the clinical extent of sensory loss, nerve damage, motor nerve conduction velocities, lepromin skin tests and immunological parameters were not distinguishable in the mononeuritic and polyneuritic groups. Therefore, all the 16 patients were included in the broad neuritic group. It was also considered more pertinent to take healthy contacts from non-endemic areas as a control group, in order to minimize non-quantifiable environmental effects of mycobacterial load of the endemic regions.

In general, normal ratios of T and B cells were found in the peripheral blood of the neuritic group. The mean and median values of T cells were marginally decreased but this reduction was not statistically significant. The functional ability of the peripheral lymphocytes to respond to general T cell mitogens showed a wide range in the patients. Though median stimulation indices appeared lower than those found in the control group, statistical significance could not be demonstrated. Clinical reassessment of the patients did not show any differences between the 'high' and 'low' responders to T cell mitogens. Poor nutritional status was not responsible for the decreased T cell functions. The 3 patients who had been treated for 5 years with dapsone showed low mitogenic stimulation (Con A 17 to 25; PHA 4.2 to 25.7). This may be related to the suppressive effect of dapsone on lymphocyte transformation as reported by Sengupta *et al* (1978).[28]

The predominant feature noted in the neuritic patients was the selective lack of *M. leprae* stimulated *in vitro* responses of peripheral lymphocytes. The degree of unresponsiveness corresponded to the values obtained in lepromatous leprosy patients of the same ethnic group (Nath *et al*, 1977);[12] and was significantly lower than the values obtained in the healthy contacts who were studied in parallel ($p < 0.002$). Repeated testing in some patients continued to show low responsiveness to *M. leprae.* It is evident that the lymphocytes from these patients are capable of responding to a cross-reacting 'recall' antigen such as PPD (Fig. 1).

The antigen specific unresponsiveness in neuritic leprosy may be due to (a) *The type of antigen used*: It had been shown in patients with reactions that sonicated bacilli gave enhanced responses in lymphocyte transformation test due to the possible release of 'cytoplasmic' antigens (Bjune *et al*, 1976).[27] In our study autoclaved bacilli were also expected to release multiple antigens. The type of antigen used may not be the cause of the observed unresponsiveness as the same batch of antigen was proved to be a good stimulant in contacts

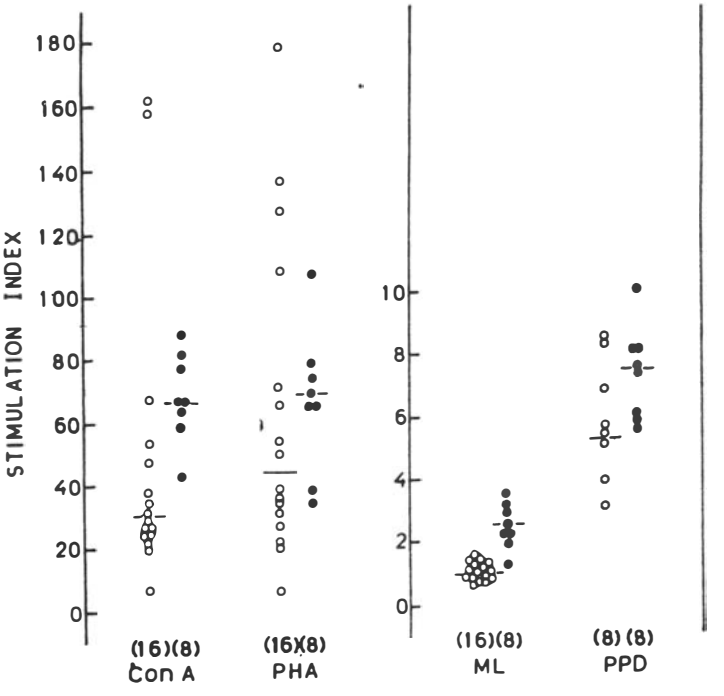

Figure 1. Lymphocyte transformation to Concanavalin A (Con A), Phytohaemagglutin (PHA), *M. leprae* and purified protein derivative (PPD) in 16 primary neuritic patients (○) and 8 healthy contacts (●).

Median Stimulation Index for each group are shown (———); Mann Whitney U Test analysis showed $p < 0.002$ only for *M. leprae* stimulation.

and tuberculoid patients who were tested in parallel. (b) *Serum Factors*: It was considered possible that suppressor factors may be present in the patients' serum. Lymphocytes from the neuritic group were washed repeatedly and cultured in pooled AB serum which had been tested for its ability to support lymphocyte transformation: (c) *Extent of Nerve Involvement*: No correlation was found between the extent of neural damage and antigen responsiveness. Both *in vivo* and *in vitro* antigen responses were lacking in our patients.

The reasons for the specific loss of antigen stimulation in this form of leprosy are not clear from the present study and need further elucidation. *M. leprae* resides within the Schwann cells of the nerves and this may form a 'protected' site which precludes contact with circulating lymphocytes. No information is available regarding the presence or absence of *M. leprae* antigen on the surface of infected Schwann cells to assess the mode of antigen presentation to specific lymphocytes. Active suppressor mechanisms involving a subclass of T cells or macrophages may be responsible for the observed inhibition of *M. leprae* responses (Gershon, 1975).[29]

Depletion of antigen reactive lymphocytes from the peripheral blood of the neuritic patients seems the most likely explanation. It may be inferred from

earlier reports of variable lepromin reactivity (Dharmendra, 1978;[3] Job, Victor & Chacko, 1977)[30] and the presence of lymphocytes in the nerve biopsies of our patients and those of others (Job *et al*, 1977)[30] that antigen specific lymphocytes are generated in neuritic leprosy. It is possible that such cells are selectively diverted to antigenic sites in the nerves thus leading to a depletion in the peripheral blood.

The patients studied by us appeared to have a stable form of the disease and did not show reactions at the time of examination. It is thus not possible to know whether the immunological enhancement noted in the neuritis due to reactions in borderline leprosy (Barnetson, 1978)[4] is also applicable in Primary Neuritic Leprosy. The selective loss of *in vitro* responsiveness to *M. leprae* seems a long-lasting phenomenon as observed in 3 patients who had been with dapsone for 5 years.

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