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EVALUATION OF CELL MEDIATED IMMUNE RESPONSES IN UNTREATED CASES OF LEPROSY

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SUMMARY

Twenty-three leprosy patients have been studied in an endemic area before institution of chemotherapy. These were comprised of ten lepromatous leprosy, four borderline lepromatous and nine tuberculoid leprosy cases on basis of clinical features, bacteriological and morphological indices. Histopathology of skin biopsies classified two as truly polar lepromatous leprosy (LL) and three as polar tuberculoid (TT), while the remaining eighteen were at various stages of evolution towards lepromatous or tuberculoid end of the spectrum. All lepromatous and borderline leprosy patients showed negative delayed hypersensitivity reaction with lepromin, but six out of fourteen patients in this category gave positive reaction with PPD. Blast transformation with PHA of peripheral leucocytes from all cases of lepromatous leprosy cultured in standard AB serum was depressed in comparison with cells from normal controls. ^{3}H-thymidine incorporation in DNA of leucocytes in presence of leprolin was lower in cells of lepromatous leprosy group as compared to those from tuberculoid and borderline cases. There was lack of production of macrophage aggregation factor in all except one case of lepromatous leprosy while the test for this factor was positive for most of the tuberculoid leprosy patients. The homing characteristics of lymphocytes tagged with ^{51}Chromium into liver and spleen of test mice were altered from the normal pattern in a large number of leprosy cases.

INTRODUCTION

Although evidence is available to indicate the possible impairment of cell mediated immune responses (CMI) in patients suffering from leprosy, observations of various investigators are not entirely coincident on this issue. Furthermore the nature of impairment is far from

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clear nor is it established whether the depression of CMI is of a general type or specifically limited to *Mycobacterium leprae* antigens. It is observed that rejection of homograft skin is delayed in lepromatous leprosy (Job & Karat cited by Hart & Rees, 1967). Histological appearance of lymph nodes of patients with lepromatous leprosy show depletion of lymphocytes in the paracortical areas and an increase in reticulohistiocytes (Turk & Waters, 1968) akin to that seen in animals treated with antileucocyte serum (ALS) (Turk & Willoughby, 1967). However, such an appearance would not necessarily indicate a deficiency of thymus-derived (T) cells, as mechanical obstruction in circulation of lymphocytes would lead to a similar histological appearance (Gaafer & Turk, 1970). A large number of patients with lepromatous leprosy show inability to develop contact sensitivity to picrylchloride and dinitrochlorobenzene (DNCB) (Bullock, 1968; Waldorf *et al.*, 1966). However, subjects showing lack of sensitization to DNCB were found to respond normally to a strong immunogen like keyhole-limpet haemocyanin (KLH) (Turk & Waters, 1969). Variable results have been obtained on the mitotic response of peripheral leucocytes of leprosy patients to mitogens (Dierks & Shepard, 1968; Bullock, 1968; Nelson, 1971). Mehra, Talwar, Balakrishnan & Bhutani (1972) observed that the transformation of peripheral leucocytes with carefully graded doses of phytohaemagglutinin (PHA) was essentially within the normal range in subjects with tuberculoid leprosy and in chronic cases of lepromatous leprosy under treatment with diaminodiphenyl sulphone (DDS). The latter showed frequently more extensive transformation than normals. It was significantly depressed though not absent, in untreated cases of lepromatous leprosy. These studies pointed to the important difference that chemotherapeutic treatment could make in the response of patients. As transformation of peripheral leucocytes with PHA alone is not necessarily a conclusive test for assessment of cell mediated immunity, it was considered appropriate to undertake more comprehensive studies on a group of freshly detected untreated cases of leprosy patients in an endemic area. The object of these studies was to enquire whether lymphocytes engaged in cell mediated immunity (CMI) are in any way functionally deficient. Immunological studies have included the delayed skin reactivity to specific *Mycobacterium leprae* antigen lepromin, and to a related mycobacterial antigen PPD; blast transformation of peripheral leucocytes with general mitogens (PHA) and specific antigens (leprolin); homing characteristics of ^{51}Cr-labelled leucocytes and the ability of the peripheral leucocytes to produce biologically active products when cultured with *M. leprae* antigens.

MATERIALS AND METHODS

Patients

The study was partly conducted in the Leprosy Control Programme Clinic run by the Danish Save the Children Organisation at Aska (Orissa) and Pogiri (Andhra Pradesh) (courtesy Dr J. de Vries and Mr Ostergaard). In all twenty-three cases of untreated leprosy patients and ten normal subjects (controls) were examined. The patients were classified on basis of clinical findings, skin smears (bacterial index and morphological index) and biopsy criteria. Biopsies of active skin lesions were fixed in 10% formalin subjected to routine processing and stained with haematoxylin—eosin and Ziehl–Neelsen technique for acid fast bacilli. Criteria for classification were those of Ridley & Jopling (1966). This classification divides

patients into five groups, namely polar lepromatous (LL), polar tuberculoid (TT), 'pure' borderline or dimorphous (BB) and subpolar lepromatous BL and tuberculoid (BT).

Delayed hypersensitivity

All patients were examined for reactions to lepromin and PPD. PPD (B.C.G. Laboratories, Madras) was given intradermally at a concentration of 10 IU in 0·1 ml. Lepromin was prepared by the method of Dharmendra (Dharmendra, 1967). The concentration was adjusted to contain 160×10^6 bacilli per ml. The dose given was 0·1 ml intradermally.

Mycobacterial antigens (Leprolin)

These were prepared from biopsies of untreated lepromatous leprosy patients made available by the Japanese Leprosy Mission for Asia, Agra, India (courtesy Dr M. Miyazaki). The bacilli were extracted from the biopsies by treatment with 0·25% trypsin in Hanks' balanced salt solution. The enzyme-treated suspension of the tissue was centrifuged at 3500 *g* to collect the mycobacteria. The pellet was washed thrice with Hanks' balanced salt solution (HBSS) and finally suspended in this medium. The mycobacteria were inactivated by exposure to UV radiations. The killed bacilli were resuspended in HBSS and adjusted to a concentration of 10×10^6 bacilli/ml according to Hanks & Lechat (1964).

Lymphocyte transformation

Twenty millilitres of blood was taken from the antecubital vein in heparinized syringes. Heparin was used at a concentration of 20 IU per ml of the blood drawn. Blood was allowed to stand at 37°C for 30 min, the plasma transferred to a tube and centrifuged at 1200 *g* for 15 min. The pellet was washed twice with medium 199. The leucocyte concentration was adjusted in a manner so as to contain 2×10^6 cells/culture tube. Medium 199 was supplemented with 20% heat inactivated (56°C, 30 min) standard AB Serum. Lymphocyte transformation was studied with specific mycobacterial antigens (10×10^6 bacilli/culture tube) as well as with a general mitogen (PHA). The experimental details for mitotic response to PHA have been described elsewhere (Mehra *et al.*, 1972). Similar procedure was followed for transformation studies with leprolin.

Macrophage aggregation factor

The supernatants of leucocytes (LCS) cultured in presence of mycobacterial antigens were collected after 36–48 hr of culture. The LCS pooled from four to five culture tubes of leucocytes of the same patient were dialysed against 0·15 M NaCl for 24 hr. The dialysate was concentrated to 1/10 of the original volume by freeze drying in a Virtis lyophylizer and filtered through Millipore nitrocellulose filter discs (0·45 μm pore size). The biological activity was tested by the macrophage aggregation test as described by Lolekha, Dray & Gotoff (1970).

Homing of ^{51}Cr-labelled lymphocytes

Twenty millilitres of blood was withdrawn as described for lymphocyte transformation. The plasma was aspirated and passed through sterile cotton columns to purify the lymphocytes. The cotton columns along with plasma were incubated for 1/2 hr at 37°C and squeezed through with medium 199. The lymphocytes collected in this way were centrifuged at 1200 *g* for 15 min and the resulting pellet of cells washed twice with medium 199. The cells

were treated with 1 ml of Tris-ammonium chloride solution (0·83%) to remove erythrocytes and washed twice with the medium 199 to remove traces of ammonium chloride. Lymphocytes were finally adjusted to a concentration of 10^7 cells per ml in medium 199 containing 20% heat inactivated foetal calf serum.

Ten micra curie of ^{51}Cr (Bhabha Atomic Research Centre, Bombay specific activity 6·7 Ci/m mole), was added to the lymphocyte suspension that was incubated for 30 min at 37°C. The cells were washed twice with non-radioactive sodium chromate solution (1%) followed twice with medium 199 and finally suspended in 0·2 ml of medium 199.

Strain A mice were injected with 0·2 ml of the lymphocyte suspension (10^7 cells) through the tail vein. After a lapse of 4 hr the liver and spleen were removed and transferred to standard size tubes. The radioactivity in the organs was measured in a Nuclear Chicago well type Counter. Lymphocytes from each patient were tested in two to three mice.

RESULTS

Clinical material

Out of a total of twenty-three patients examined, ten were lepromatous leprosy cases on the basis of clinical features, bacteriological and morphological indices. The histopathological report on biopsies indicated two out of them as truly polar lepromatous leprosy cases, four as borderline lepromatous (BL), two indeterminate; and two as BB on Ridley and Jopling scale. Four patients were classified clinically as well as by histopathological criteria as borderline (BB) cases. The remaining nine were clinically tuberculoid and were bacteriologically negative. Histopathologically three of them were classified as TT, four as BT, one as indeterminate and one as BB. In short the majority of cases encountered in the field control centres at the time of their first detection did not belong to the frankly polar LL and TT forms but represented a substantial proportion of intermediate category patients who could, however, be progressing towards lepromatous or tuberculoid ends of the spectrum.

Delayed hypersensitivity skin reactions

All cases in the clinically lepromatous group gave negative lepromin reaction when read after 3 weeks of intradermal injection, while all but one of the clinically assessed tuberculoid leprosy group patients gave positive reaction with lepromin. The borderline group (BB) were lepromin negative. Anergy to delayed hypersensitivity skin reactions was not a generalized trait of the lepromatous leprosy patients. Four patients with lepromatous leprosy gave positive reactions with PPD, even though they were lepromin negative, suggesting the selective inability of a fraction of lepromatous leprosy patients to manifest cell mediated immune responses (CMI) to mycobacterium leprae antigens, while CMI to antigens of another acid fast mycobacterium was normal. However, six out of ten patients in this group gave negative reaction to both lepromin and PPD. These observations would argue for both specific and nonspecific depression of CMI in leprosy depending perhaps on the stage of progression of the disease (or other factors).

Blast transformation

Lymphocytes from all cases of lepromatous leprosy showed a diminished response to PHA ranging from 26 to 73% of the response given by lymphocytes from normal subjects.

It may be stated that standard AB serum was used for cultures of leucocytes of contro and leprosy patients in order to minimize the possible influence of factors present in the sera of leprosy patients on the transformation processes. The transformation with mycobacterial antigens was also depressed in all or majority of lepromatous leprosy cases. In the tuberculoid category, the transformation with PHA is not impaired as has been reported elsewhere (Mehra *et al.*, 1972); transformation with leprolin was much better than was the case of lymphocytes from subjects with lepromatous leprosy. In the borderline category patients (Table 2), the pattern was of a dispersed type. (Tables 1, 2 and 3).

Macrophage aggregation test (MAF)

This test was carried out to assess the ability of lymphocytes of various categories of patients to produce biologically active polypeptides in presence of *M. leprae* antigens. The results were by and large parallel to the indications given by the blast transformation tests. In all except one of lepromatous leprosy cases, low MAF was produced (Table 1), whereas tuberculoid leprosy cases gave positive MAF reaction.

Distribution of ^{51}Cr-labelled lymphocytes

The deficient performance of lymphocytes from lepromatous leprosy cases could be due to several reasons. Amongst these would be the possible alterations of surface characteristics of these cells by cytotoxic factors, antibodies or other agents. Martin & Miller (1969) have described a simple approach in which the interaction of antileucocyte serum with the lymphocytes can be monitored by a marked change in the distribution pattern of these cells in the liver and spleen of mice. Peripheral lymphocytes of the subjects under study were tagged with ^{51}Cr and were examined for their homing characteristics in the liver and spleen of mice. The distribution of radioactivity due to these cells in the two organs indicated by the ratio of the counts in liver and spleen from control subjects was of the order of 1·1:1. In seven out of ten lepromatous leprosy cases, the homing characteristics of the leucocytes were modified in a manner so that the liver to spleen ratio was much higher than the controls. This could not, however, be taken as a consistent phenomenon for patients of this category, as leucocytes from two lepromatous leprosy cases had a distribution akin to that of the normals.

DISCUSSION

It is becoming apparent that the different types of leprosy are not always stable. Patients with lepromatous leprosy can show stigmata of a previous borderline or dimorphous phase, and, left untreated, in some patients the disease will deteriorate towards lepromatous as it extends. Probably for this reason no single test can be adequate to assess faithfully the immunological status of the patient. These variations would be inherent not only because of the limitations of the reply furnished by a test but also by the state of host-mycobacterium interaction, at which the test is carried out. It is likely that the immunological responsiveness gets modified in the process of evolution of the disease.

Amongst the deductions permissible from this study, subjects with lepromatous leprosy show both specific and non-specific anergy to mycobacterial antigens in delayed hypersensitivity reactions. The blast transformation of peripheral leucocytes with graded doses of PHA, and with leprolin are depressed. The leucocyte-macrophage interaction is impaired

TABLE 1. Lepromatous leprosy cases

S. No.	Code No.	Name	Clinical status	Histo-pathology	Bacterial index and morphological index	Lepromin test		PPD	Blast transformation		MAF	^{51}Cr‡ distribution L/S ratio
						Early	Late		PHA*	Leprolin†		
1	11	KM	L	LL	+++11·8%	−ve	−ve	15 mm	73·4	156	−ve	6·2:1
2	1	TN	L	LL	++++ 6·4%	15 mm	−ve	15 mm	27·9	73	−ve	2·2:1
3	2	DS	L	BL	++++ 1·6%	9 mm	−ve	−ve	37·8	122	−ve	5·6:1
4	25	BM	L	BL	+++14·3%	−ve	−ve	−ve	48·6	132	−ve	1·5:1
5	5	KM	L	BL	++++ 2·4%	−ve	−ve	−ve	26·5	72	−ve	1·3:1
6	4	KP	L	BB-BL	++++ 1·4%	10 mm	−ve	−ve	32·5	86	−ve	21·4:1
7	10	GS	L	Indeter-minate	+++ 1%	−ve	−ve	−ve	68·9	81	−ve	10·2:1
8	26	DN	L	Indeter-minate	++ 8·3%	−ve	−ve	−ve	49·1	N.D.	++++	3·4:1
9	6	MN	L	BB	++++ 2·4%	11 mm	−ve	13 mm	31·7	205	−ve	1·1:1
10	3	GB	L	BB	+++ 3%	12 mm	−ve	20 mm	33·2	35	−ve	5·2:1

* Percent relative response: $\frac{\text{Cpm}/2\times10^6 \text{ cells of the patient}}{\text{Cpm}/2\times10^6 \text{ cells of control subjects}} \times 100$—Cpm for leucocytes from normal subjects cultured with PHA were of the order of 14000.

† Cpm/2×10^6 cells: Cpm/2×10^6 cells from normal subjects cultured with Leprolin were 349,303,315 for three cases investigated.

‡ Liver to spleen distribution of ^{51}Cr leucocytes from normal subjects was 1·1:1. The values are mean of results on two to three mice for each batch of leucocytes.

N.D. = Not done.

TABLE 2. Borderline lepromatous leprosy cases

S.No.	Code No.	Name	Clinical status	Histo-pathology	Bacterial index and morphological index	Lepromin test		PPD	Blast transformation		MAF	^{51}Cr‡ distribution L/S Ratio
						Early	Late		PHA*	Leprolin†		
1	12	SK	BB-BL	BB	++++12·4%	9 mm	−ve	−ve	62	448	+++++	N.D.
2	13	BM	BL	BB/BT	++ 1·6%	10 mm	−ve	18 mm	43·2	387	+++++	2·2:1
3	14	KD	BB	BB	++ 1·6%	−ve	−ve	18 mm	88·4	213	+++++	5·9:1
4	15	NP	BB	BB/BT	++ 3·8%	11 mm	−ve	−ve	ND	ND	−ve	3·4:1

* Percent relative response: $\frac{\text{Cpm}/2\times10^6 \text{ cells of the patient}}{\text{Cpm}/2\times10^6 \text{ cells of control subjects}} \times 100$ − Cpm for leucocytes from normal subjects cultured with PHA were of the order of 14000.

† Cpm/2×10^6 cells: Cpm/2×10^6 cells from normal subjects cultured with Leprolin were 349,303,315 for three cases investigated.

‡ Liver to spleen distribution of ^{51}Cr leucocytes from normal subjects was 1·1:1. The values are mean of results on two to three mice for each batch of leucocytes.

N.D. = Not done.

TABLE 3. Tuberculoid leprosy cases

S. No.	Code No.	Name	Clinical status	Histo-pathology	Bacterial index and morphological index	Lepromin test		PPD	Blast transformation*		^{51}Cr† distribution L/S Ratio
						Early	Late		Leprolin	MAF	
1	20	BG	T	TT	−ve	5 mm	5 mm	15 mm	565	++++	N.D.
2	24	KN	T	TT	−ve	11 mm	14 mm	−ve	N.D.	+++	2·4:1
3	23	KS	T	TT	−ve	−ve	−ve	−ve	−ve	++++	1·7:1
4	21	GB	T	BT	−ve	16 mm	16 mm	N.D.	450	+++++	N.D.
5	22	NA	T	BT	−ve	−ve	15 mm	14 mm	385	+++	7·1:1
6	17	PN	T	BT	−ve	9 mm	12 mm	−ve	175	++++	10·4:1
7	18	KP	T	BT	−ve	7 mm	8 mm	−ve	N.D.	N.D.	N.D.
8	16	MP	T	BB (Indeter)	−ve	7 mm	4 mm	−ve	176	+++	5·3:1
9	19	DB	T	BB	−ve	11 mm	12 mm	−ve	148	++	N.D.

* Cpm/2 × 10^6 cells: Cpm/2 × 10^6 cells from normal subjects cultured with Leprolin were 349, 303, 315 for three cases investigated.

† Liver to spleen distribution of ^{51}Cr leucocytes from normal subjects was 1·1:1. The values are mean of results on two to three mice for each batch of leucocytes.

N.D. = Not done.

by virtue of the inability of peripheral leucocytes of lepromatous leprosy patients to produce biologically active peptides such as macrophage aggregation factor (MAF).

The nature of defect in lymphocyte function is not clear nor is it known at what stage it develops. The abnormal behaviour of leucocytes from many lepromatous leprosy cases in terms of their homing characteristics in various organs is a pointer to possible alterations in the surface characteristics of these cells.

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