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Mycobacterium leprae reactive T cell clones from lepromatous leprosy patients after prolonged dapsone chemotherapy

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Summary The proliferative responses of peripheral blood mononuclear cells (PBMC) to *Mycobacterium leprae* and BCG were studied in two groups of leprosy patients: a group of 8 lepromatous patients who had been on treatment for more than 20 years (TLL) and a group of 8 untreated lepromatous leprosy patients (ULL). The mean response to *M. leprae* of the TLL group was 6195 cpm with 5 of the 8 patients responding positively. The mean response to *M. leprae* of the ULL group was 617 cpm, with only 1 patient showing a positive response. The corresponding proliferative responses to BCG were 19,908 cpm in the TLL group and 7908 in the ULL group.

Thirteen *M. leprae* reactive clones were established from 2 TLL patients and 5 *M. leprae* reactive clones were established from 2 tuberculoid leprosy patients. Seven of these clones, 4 from the TLL patients and 3 from the tuberculoid (TT) patients could be studied further. Three of the TLL clones responded specifically to *M. leprae*, while one of the clones exhibited a broad cross-reactivity to other mycobacteria. All of these clones were of the CD4+CD8− phenotype.

Our findings suggest that responsiveness to *M. leprae* can be detected *in vitro* in a proportion of LL patients who have undergone prolonged chemotherapy, and that this response involves *M. leprae* reactive CD8+CD8− T cells, of which some appear to be specific to *M. leprae*.

Introduction

It is well documented that lepromatous leprosy patients do not respond to *M. leprae*

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antigens in lymphoproliferative assays.[1-3] This nonresponsiveness has been reported to persist in lepromatous patients who have been on chemotherapy for more than 10 years.[4] Furthermore, the degradation of *M. leprae* in host tissue appears to take place very slowly.[5] Thus an investigation of this nonresponsiveness, particularly in relation to its duration and its possible reversal, would require the study of patients who had undergone even longer periods of chemotherapy. A unique opportunity for such a study exists in Sungai Buloh where one of the most comprehensive of the earlier trials of dapsone has been conducted.

In this study, a small number of LL patients, who had undergone more than 20 years of chemotherapy, were screened for responsiveness to *M. leprae* in proliferation assays. Some of these long-treated patients were found to respond to *M. leprae*. In addition, T-cell clones which recognize *M. leprae* could be raised from some of these patients using standard techniques.

Materials and methods

PATIENTS

Untreated and long-treated (20 years) LL patients, classified according to the criteria of Ridley & Jopling,[6] were the main subjects of this study. Tissue slides of the long-treated patients (TLL) were re-examined (by DSR) independently of the immunological results and only those classified as having lepromatous leprosy were included in this study. Most of the long-treated patients were residents of Sungai Buloh, while the untreated patients were new cases in the hospital awaiting classification and treatment. Two TT patients were also included in the study. Patients whose T cells were considered suitable for cloning were informed about the procedure and told that they would be bled twice a month for the initial period of the study. The most acceptable arrangement was to take 20–30 ml of blood from the subject once a fortnight with allowances for rest periods when the need for PBMC was not so critical.

LYMPHOCYTE TRANSFORMATION TEST

Mononuclear cells were separated from the patient's peripheral blood by density centrifugation on a Ficoll/Hypaque gradient (Pharmacia, Sweden). The peripheral blood mononuclear cells (PBMC) were cultured at a concentration of 10^5 cells/well, in 96 well U-bottom trays in the presence of antigens. The trays were incubated for 6 days at 37°C in a humidified atmosphere of 5% CO_2 in air. On the 6th day, the cultures were pulsed with 0·045 MBq. ^{3}H-thymidine (specific activity $= 185 \times 10^3$ mol^{-1}) for 4 hours, after which they were harvested with a Skatron harvester (Norway). The radioactivity incorporated was determined by liquid scintillation spectroscopy. The results were expressed as the mean value of counts per minute (cpm) of triplicate cultures. A subject was considered a responder to a given antigen when cpm Δ cpm = (cpm of PBMC + Ag) − (cpm of PBMC) > 2000 and T/C = (cpm of PBMC + Ag)/(cpm of PBMC) > 2.

M. LEPRAE INDUCED T CELL CLONES

The procedure for raising *M. leprae* reactive T cell clones has been described elsewhere.[7]

Briefly, 20×10^6 peripheral blood mononuclear cells were cultured in complete medium (RPMI 1640 + 10% AB serum + 50 μg/ml gentamycin) with 10 μg/ml soluble *M. leprae* antigen (Batch CD 52) in 25 cm^3 flasks (Costar). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 6 days. On the 6th day the cells were washed and recultured with fresh autologous irradiated (2200 rad) feeder cells and antigen. Three days later the cells were washed and cloned in 60-well Microtest plates (Nunc) by limiting dilution. After 10–12 days cells from positive wells were transferred to 96-well flat-bottomed trays (Nunc) with fresh irradiated autologous feeders + antigen + recombinant interleukin-2 (IL-2, Cetus). Then, 3–5 days later, growing cultures were transferred to 24-well plates (Nunc) with 1×10^6/ml autologous irradiated feeder cells + 10 μg/ml of soluble *M. leprae* antigen + 100 U recombinant IL-2. Recombinant IL-2 and fresh medium were added on day 3. This cycle was repeated every 7th day until there were enough cells for study.

ANTIGEN INDUCED PROLIFERATION OF T CELL CLONES

To test the antigen reactivity of the clones in a proliferative assay, adherent cells from 10^5 irradiated PBMC were added to each well of a 96-well flat-bottomed tray as antigen-presenting cells. 10^4 cloned T cells and antigens at optimal concentration were also added. Antigens were added in triplicate. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. On the 3rd day the cultures were pulsed with 0·045 MBq ^{3}H-thymidine for 4 hours, after which they were harvested with a Skatron harvester (Norway). The results were expressed as the mean value of cpm of each triplicate. A clone was considered a responder to a given antigen when Δ cpm = (cpm of cultures with T-cell clone + antigen + APC) − (cpm of cultures with T-cell clone + APC) > 1000 and T/C = (cpm of cultures with T-cell clone + antigen + APC)/(cpm of cultures with T-cell clone + APC) > 2.

ANTIGENS

The *M. leprae* antigens, soluble (CD 60 and CD 52) and whole (CD 46), were provided by Dr R J W Rees (Mill Hill, London), from the IMMLEP (WHO) *M. leprae* Bank. BCG and PPD were obtained from the Statens Serum Institute, Copenhagen, Denmark. The following soluble antigens, Scrofulin, Kansasin, Aviumin, Flavescin, Xenopin, Duvalin, Nonchromogencin, Smegmatin, Vaccin, Gordonin, Phlein, Tuberculin and Mw, made by the ultrasonication of a panel of fast- and slow-growing mycobacteria, were kindly provided by Dr J L Stanford and Dr G Rook, Middlesex Hospital Medical School, London, U.K.

Results

Peripheral blood mononuclear cells (PBMC) from 8 clinically, bacteriologically and histopathologically classified lepromatous leprosy patients, who have not yet been treated (ULL) and from 8 long-treated (> 20 years), clinically improved and bacteriologically negative lepromatous leprosy patients (TLL) were tested for *in vitro* proliferation to *M. leprae* and BCG antigens (Figure 1). The mean response to *M. leprae* in the TLL group was 6195 cpm with 5 of the 8 patients responding positively. The mean response to *M.*

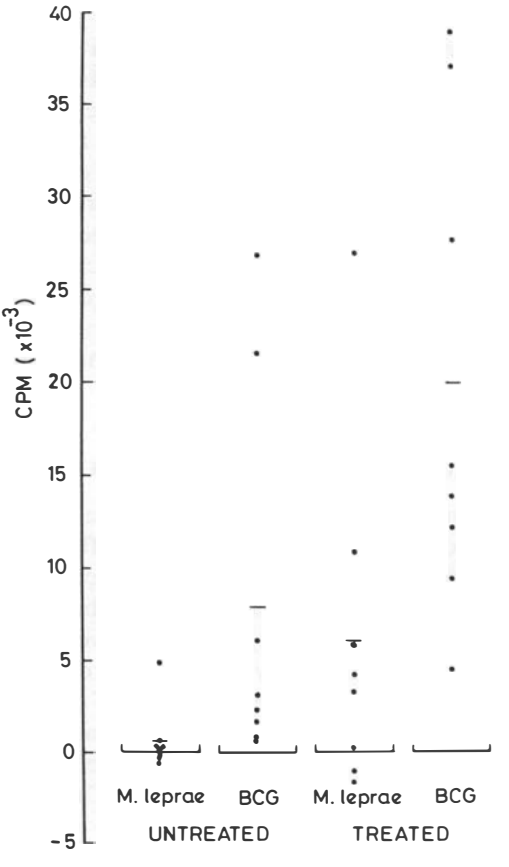

Figure 1. Proliferative responses to BCG and *M. leprae* in ULL and TLL patients. Each dot represents the proliferative response of one patient which is expressed in mean cpm of triplicate cultures. Horizontal bars represent the mean response of the group.

Table 1. Preliminary screening of antigen reactivity of T cell clones from TLL patients

Patient	LSH				LLH					
Clone	2/4D	5/4C	7/5E	8/7D	2/1C	3/3B	2/9D	2/7C	4/1D	6/6A
Antigen										
Control	1254	738	755	461	421	731	370	180	288	314
CD 52	6821	5919	2961	3691	40746	23487	24596	26971	14918	5215
CD 46	201	229	681	390	713	666	255	389	425	154
BCG	504	713	1265	6202	699	2725	5508	661	687	673
PPD	326	419	637	9624	1419	647	2061	1779	546	308

CD 52 is a soluble *M. leprae* antigen.
CD 46 is a whole *M. leprae* antigen.
The results are expressed in mean cpm of triplicate cultures.
Positive responses are underlined.

Table 2. Antigen reactivity and surface phenotype of T cell clones from TLL and TT patients

	TT			LL			
Patient	EL/1	SB/1	SB/2	LSH 5/4C	LSH 2/4D	LLH 6/6A	LLH 2/9D
Control	312	983	375	921	606	895	191
CD 52	5358	7138	13680	5502	12437	6090	18007
CD 46	1193	405	638	621	893	605	205
BCG	493	436	307	491	1089	444	1459
PPD	331	277	657	366	1493	586	1967
Scrofulin	328	348	695	487	524	790	2170
Kansasin	587	285	548	190	282	639	98
Aviumin	325	268	268	285	443	1421	1553
Flavescin	646	361	392	171	450	421	2055
Xenopin	336	372	412	446	396	1620	1382
Duvalin	421	530	327	448	482	321	233
Nonchromogencin	310	209	165	198	216	459	217
Smegmatin	457	278	272	157	291	427	144
Vaccin	283	150	343	369	253	709	4716
Gordonin	380	191	391	238	175	537	4413
Phlein	40	245	259	677	401	1272	208
Tuberculin	808	247	327	474	437	585	5219
Mw	744	372	284	380	663	1476	5179
Phenotype CD4	+	+	+	+	+	+	+
CD8	−	−	−	−	−	−	−

Results are expressed in mean cpm of triplicate cultures.
Positive responses are underlined.

leprae of the ULL group was 617 cpm, with only 1 patient showing a positive response. The difference in response to *M. leprae* between the two groups was not statistically significant ($p > 0{\cdot}3184$, Wilcoxon Rank Sum Test). The corresponding proliferative responses to BCG were 19,908 cpm in the TLL group and 7908 in the ULL group.

To determine the characteristics of the responding cells from the TLL patients, 13 T cell clones, against *M. leprae*, were successfully raised from the PBMC of 2 of these 8 patients. For comparison five *M. leprae* induced T cell clones were also raised from 2 tuberculoid leprosy patients. In the preliminary screening of the TLL clones, 6 clones were found to be specific, 4 clones were cross-reactive and 3 clones were non-reactive (Table 1). All of the 5 clones raised from the TT patients were found to be specific for *M. leprae* in the preliminary screening.

Seven of these clones, 4 from the TLL patients and 3 from the TT patients, could be studied further by assessing their proliferative responses to soluble antigens from a panel of 13 cultivable mycobacteria (Table 2). Three TLL clones responded specifically to *M. leprae*, while one clone exhibited broad cross-reactivity, with responses to various other mycobacteria. All 7 of the T cell clones were of the CD4+CD8− phenotype (Table 2).

Discussion

There is a good correlation between the *in vitro* proliferative response and the *in vivo* cell mediated immunity throughout the clinical spectrum of leprosy.[2] The *in vitro* response to

M. leprae has been found to be negative in untreated LL patients. Furthermore, earlier studies have suggested that the cell mediated immune response to *M. leprae* remains negative even after prolonged treatment.[4] However, such studies have been time limited as the first effective chemotherapeutic agent, dapsone, was only introduced in the late 1940s. Sungai Buloh is one of the few places which made an early start in assessing the efficacy of dapsone for the treatment of leprosy. Patients from these trials have been on prolonged treatment and were carefully monitored throughout and after the period of treatment. They thus represent a unique source of study especially for the effects of prolonged treatment.

In this study we were able to examine the responses of 8 such long-treated patients and to compare them with those of untreated LL patients. Five of the long-treated patients showed a positive response to *M. leprae* so that the mean response to *M. leprae* of the TLL group was 6195 cpm. The mean response to *M. leprae* in the ULL group was 617 cpm, with only 1 patient showing a positive response. It is possible that some of these long-treated patients had at some stage passed through a responder phase such as a borderline tuberculoid phase. This idea is supported by the fact that several of these patients were classified as LLs. The others could not be classified further than LL, either for technical reasons or because the lesions were resolving. There were no definite cases of the primary LLp group, a rarity in Malaysia. In an earlier study, LL patients (bacteriologically negative for 8–20 years) who were immunized with *M. leprae* or *M. leprae* and BCG failed to develop a delayed type hypersensitivity to *M. leprae*.[8] This was attributed to antigen persistence. It is thus interesting that immunological responsiveness should now be demonstrated in a similar group of patients who have undergone an even longer duration of treatment. There is also evidence that patients similar to ours, in some cases, do regain their lepromin positivity (Dr M F R Waters, personal communication).

The establishment and study of T cell clones from these long-treated LL patients revealed that their ability to respond to *M. leprae* is due to cells of the CD4+ phenotype. The establishment of CD4+ clones from tuberculoid patients have been reported previously.[9,10] More recently there have been reports of CD8+ *M. leprae* reactive T cell clones raised from a borderline lepromatous leprosy patient[11] and from the lesions of a lepromatous leprosy patient.[12] To the best of our knowledge, this is the first report of *M. leprae* reactive CD4+ T cell clones isolated from the PBMC of lepromatous leprosy patients. These clones display response patterns similar to the other *M. leprae* reactive CD4+ T cell clones that have been isolated from leprosy patients in that they range from a pattern of strict specificity to patterns of broad cross-reactivity.

Our study shows that at least some lepromatous leprosy patients have the potential for generating T cell responses to *M. leprae* specific as well as *M. leprae* cross-reactive epitopes.

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