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Cellular immune response to the cell walls of *Mycobacterium leprae* in leprosy patients and healthy subjects exposed to leprosy

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SUMMARY

Cell walls of *M. leprae* consist of complex arrangements of carbohydrate, lipid, peptidoglycan and protein molecules. Recently, extractable proteins of a wide range of molecular weights were identified as components of the cell wall. We have examined the cellular immune responses of Nepali leprosy patients to a cell wall preparation of *M. leprae* enriched for these proteins. Strong lymphocyte proliferative responses to the antigens were present in half of the paucibacillary leprosy patients and in the majority of healthy control subjects with occupational exposure to leprosy. Patients with multibacillary disease responded poorly and patients with tuberculosis had intermediate responses. Proliferative responses to the cell wall protein fraction were strongly correlated to the proliferative responses to sonicates of the whole leprosy bacillus. Immunization of mice with cell wall proteins resulted in inhibition of growth of *M. leprae* following foot-pad inoculation with viable organisms. Therefore cell-mediated immune responses to the extractable proteins of the cell wall may play a role in protective immunity against *M. leprae* infection.

Keywords cell wall protein *Mycobacterium leprae* protective immunity lymphocyte proliferation

INTRODUCTION

Protective immunity to intracellular parasites such as *M. leprae* is mediated by T lymphocyte recognition of protein antigens [1]. A range of protein antigens of *M. leprae* have been characterized, including the 70-, 65-, 36-, 35-, 28-, 18- and 12-kD proteins defined by MoAbs [2–4]. Further specific gene products have been recognized by *M. leprae*-reactive T cell clones [5] and sera from leprosy patients [6,7]. Cellular recognition of the *M. leprae* 70-, 65- and 18-kD proteins occurs in leprosy as well as in tuberculosis patients. However, the pattern of hyporesponsiveness to whole *M. leprae* characteristic of lepromatous leprosy patients is not observed for these proteins [8,9] (and unpublished observations). Recently highly immunogenic cell wall associated proteins have been isolated by a series of chemical purifications from sonicates of *M. leprae* [10–12]. These were recognized by *M. leprae*-specific T cell clones isolated from a few leprosy patients, suggesting they may be dominant antigens in the host response at the site of infection [11].

Here we demonstrate strong and specific T cell responses to the extractable *M. leprae* cell wall proteins in paucibacillary leprosy patients and their contacts and the lack of such responses in multibacillary leprosy patients. Patients with active tuberculosis showed intermediate responses to cell wall proteins proportional to their responses to the whole leprosy bacillus. We also confirm that the cell wall proteins elicting these responses were able to stimulate protective immunity in mice against *M. leprae* foot-pad infection [13].

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SUBJECTS AND METHODS

Antigens

The purified cell walls of *M. leprae* were obtained by sucrose density gradient centrifugation of a sonicate of leprosy bacilli isolated from infected armadillo spleen [12]. The non-bound cell wall proteins (CWP) that are the basis of the present study were extracted with 2% SDS at 56°C. Detergent was removed, the preparation was freeze-dried for shipment and was then reconstituted in sterile PBS, vortexed vigorously and aliquots frozen at −20°C. The reconstituted preparation was not completely soluble. Whole *M. leprae* (lot CD130 at 3×10^{10} AFB/ml) and *M. leprae* sonicate (lot CD115) were kindly supplied by Dr R. J. W. Rees through the Immunology of Leprosy (IMMLEP) programme of the World Health Organization. BCG sonicate

was prepared from *M. bovis* BCG (CSL, Melbourne, Australia) [4].

Subjects

Forty-six Nepali leprosy patients (37 men, nine women; age range 15–70 years) diagnosed on clinical and bacteriological grounds according to the Ridley–Jopling classification [14] were included in this study. Two patients were diagnosed as having primary neuritic leprosy and 30 as borderline tuberculoid leprosy. These 32 patients were considered as paucibacillary (PB) leprosy. Ten borderline lepromatous and four lepromatous made up the multi-bacillary (MB) group. At the time of the study, three of the patients were previously untreated, five had been treated with dapsone montherapy alone for 1–16 years; 32 had received multi-drug therapy [15] for 1–59 months. Six patients had received both forms of therapy. Twenty-seven patients were tested for lepromin reactivity by injecting 1×10^7 heat-killed armadillo-derived *M. leprae* intradermally into the forearm and measuring the induration after 3–4 weeks. Induration of 3 mm or more in diameter was considered positive. Twenty-two patients were lepromin positive and five were lepromin negative.

Twenty-three Nepali tuberculosis (TB) patients (18 men, five women; age range 16–58 years) with active pulmonary disease confirmed by sputum microscopy and chest X-ray were also studied. At the time of testing, all had been treated with anti-tuberculosis drug therapy for 1–51 months. The TB patients were not tested with lepromin. Twelve Nepali leprosy health workers (11 men, one woman, aged 18–33 years) from Anandaban Leprosy Hospital were also tested. They had been employed in the hospital for 1–10 years. Nine were lepromin positive and one was lepromin negative. The immunological studies were approved by the Medical Research Review Panel of Anandaban Hospital.

Lymphocyte proliferation assays

Venous blood from the subjects was collected into preservative-free heparin 10 U/ml (Sigma, Poole, UK) and peripheral blood mononuclear cells (PBMC) were separated by centrifugation over a Ficoll–Hypaque gradient (SG 1.078). The cells were washed in RPMI 1640 (Sigma) containing 0·01 M sodium bicarbonate and 25 mM HEPES buffer and 2 mM L-glutamine plus 50 mg/*l* penicillin and 100 mg/*l* streptomycin. Cell suspensions were adjusted to 10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated human A serum. Aliquots of 200 μl were dispensed into the wells of a flat-bottomed 96-well microtitre plate (Flow Laboratories, Irvine, UK) and antigens diluted in RPMI 1640 added in triplicate. BCG sonicate, *M. leprae* sonicate (MLS), and the cell wall proteins (CWP) were used at final concentrations of 10, 3, 1, 0·3, 0·1 μg/ml. Whole *M. leprae* (WML) was used at final concentrations of 10^8, 10^7, 10^6 bacilli/ml, and concanavalin A at 10 μg/ml. Control wells received media only. The cultures were incubated for 5 days at 37°C in 5% CO_2 in humidified air before pulsing with ^{3}H-thymidine 0·5 μCi/well (Amersham) for 16 h. Incorporation of ^{3}H-thymidine into proliferating cells was measured in d/min by liquid scintillation spectroscopy using a Tricarb 1500 counter (Packard CT). The specific ^{3}H-thymidine incorporation was obtained by subtracting the mean d/min in control triplicate wells from the mean of the test triplicates. The stimulation index (SI) was the ratio of the mean ^{3}H-thymidine incorporation in the test wells to that in wells without antigen. A response was considered positive if the SI was ≥ 4 and the net d/min was > 2000.

Vaccination of mice against M. leprae *infection*

Swiss albino mice aged 6–8 weeks were vaccinated subcutaneously in both hind-flanks with either CWP (50 μg or 20 μg), WML (10^8 AFB), or PBS. All antigens were emulsified in a 1:1 mixture with Freund's incomplete adjuvant (CSL, Melbourne, Australia). One month later, both hind foot-pads were innoculated with 10^4 *M. leprae* using a strain derived from an untreated leprosy patient, which had been passaged through mouse foot-pads. When the growth of *M. leprae* in the foot-pads of mice vaccinated with PBS reached 10^6/foot-pad, the mice from all groups were killed and the numbers of acid-fast bacteria (AFB) in each foot-pad were counted [16].

Statistical analysis

The differences in the mean ^{3}H-thymidine incorporation in patient groups were tested by Student's *t*-test and the differences in the proportion of positive subjects in individual groups were tested by the χ^2 test with the Yates correction. The correlations between responses to any two antigens in individual subjects were tested with the Spearman rank correlation coefficient. Differences in the range of growth in different groups of mice receiving different immunization were compared with the Mann–Whitney *U*-test.

RESULTS

Lymphocyte proliferation assays

The *M. leprae* CWP induced strong proliferative responses in the majority of the healthy exposed contacts of leprosy patients and in almost half of the PB patient group. Only one of the 14 MB leprosy patients reacted. Of the TB patients, 26% demonstrated a cellular response to the antigen (Table 1). The magnitude of the responses to CWP was significantly greater in the healthy contacts and PB patients than in the MB and TB patients over the range of concentrations examined (Fig. 1).

The proliferative responses to CWP antigens were strongly related to the responses to WML and a soluble preparation of the organism (MLS) with 32 out of 33 of the CWP responders also responding to one of these two antigens. The relationship to lepromin reactivity was less clear with only 16 out of 29 (55%) of lepromin-positive patients having responses to CWP compared with CWP reactivity in two out of five (40%) of lepromin-negative subjects. Levels of response to CWP were not significantly different between lepromin-positive and lepromin-negative subjects.

Correlations

In individual subjects there were strong correlations between responses to *M. leprae* CWP and MLS ($r_s = 0{\cdot}903$) and between responses to *M. leprae* CWP and BCG sonicate ($r_s = 0{\cdot}652$). The correlations were maintained in the PB subgroup and contacts. However, there was no correlation between BCG sonicate and CWP responses in MB patients, because most (71%) were responders to BCG sonicate and only one out of 14 responded to *M. leprae* CWP (Table 2).

Table 1. Proliferative responses to BCG sonicate, *M. leprae* sonicate and *M. leprae* cell wall protein in patients with paucibacillary (PB) and multibacillary (MB) leprosy or tuberculosis (TB) and healthy control subjects exposed to leprosy

Subject group	BCG sonicate		*M. leprae* sonicate		CWP antigen	
	% positive	Mean d/min (s.e.m.)	% positive	Mean d/min (s.e.m.)	% positive	Mean d/min (s.e.m.)
Contacts ($n=12$)	83	16878 (6168)	83	6567 (2187)	83	8069 (1669)
PB ($n=32$)	69	10622 (3932)	50	10308 (3440)	47	10414 (3198)
MB ($n=14$)	71	6030 (2634)	14†	1110 (1993)†	7‡	864 (240)‡
TB ($n=23$)	74	6716 (1504)	35*	2185 (2382)*	26†	2207 (510)‡

Positive responders are defined as subjects with a SI >4 and net proliferative response $\geqslant 2000$ d/min at final concentration of 10 μg/ml.

Significant differences in the proportion of responders or in the mean proliferative response (d/min) compared with the contact group.

(* $P<0.05$, † $P<0.01$, ‡ $P<0.001$).

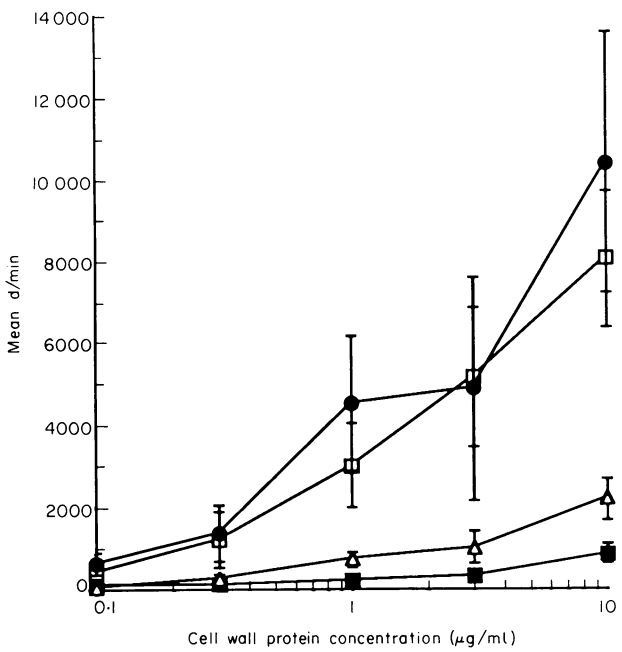

Fig. 1. Mean proliferative responses (±s.e.m.) of healthy contacts (□, $n=12$), PB (●, $n=32$) and MB (■, $n=14$) leprosy patients and TB patients (△, $n=23$) to differing concentrations of the *M. leprae* CWP. The differences between the mean response of both contacts and PB patients and responses of MB and TB patients were significant at CWP concentrations of 10 μg/ml ($P<0.001$), 3 μg/ml ($P<0.005$) and 1 μg/ml ($P<0.01$).

Table 2. Correlation of the proliferative responses in individuals to *M. leprae* CWP antigen, *M. leprae* sonicate (MLS), and BCG sonicate (BCG)

	Correlation coefficient (r_s)
CWP *versus* MLS	
All subjects	0·903 ($P<0.001$)
Contacts	0·86 ($P<0.001$)
PB	0·798 ($P<0.001$)
MB	0·553 ($P<0.05$)
TB	0·941 ($P<0.001$)
CWP *versus* BCG	
All subjects	0·652 ($P<0.001$)
Contacts	0·594 ($P<0.05$)
PB	0·799 ($P<0.001$)
MB	0·057 ($P=$NS)
TB	0·821 ($P<0.001$)

Protection of the mouse foot-pad against M. leprae *infection*

Mice that had been immunized with CWP showed a variable degree of inhibition in the growth of *M. leprae* in the foot-pad. Multiplication of *M. leprae* in mice receiving a placebo injection reached a mean level of $2{\cdot}3 \times 10^6$ *M. leprae* per foot-pad after 6 months, while in mice receiving immunization with CWP (20 μg) the mean growth was $4{\cdot}4 \times 10^5$ *M. leprae* per foot-pad ($P<0.05$). This level of protection was similar to that observed after immunization with 10^8 whole leprosy bacilli (mean growth $3{\cdot}2 \times 10^5$ per foot-pad, $P<0.05$). However, immunization with 50 μg of CWP only caused inhibition of *M. leprae* growth in some mice (mean growth $5{\cdot}5 \times 10^5$, NS) (Fig. 2).

DISCUSSION

Cell walls of bacteria have been shown to play an important role in stimulating host responses. The cell walls of mycobacteria are complex networks of covalently and non-covalently linked lipids, complex carbohydrates, proteins and peptidoglycans [17]. The *M. leprae* phenolic glycolipid-I and lipoarabinomannan molecules stimulate a strong humoral response which is maximal in patients at the lepromatous pole of the disease [18,19]. Early studies with a cell wall-enriched fraction of *M. leprae* (MLW 1) showed strong proliferative responses developed in contacts of leprosy patients and PB leprosy patients, but not in MB patients. Responses were also seen in healthy subjects

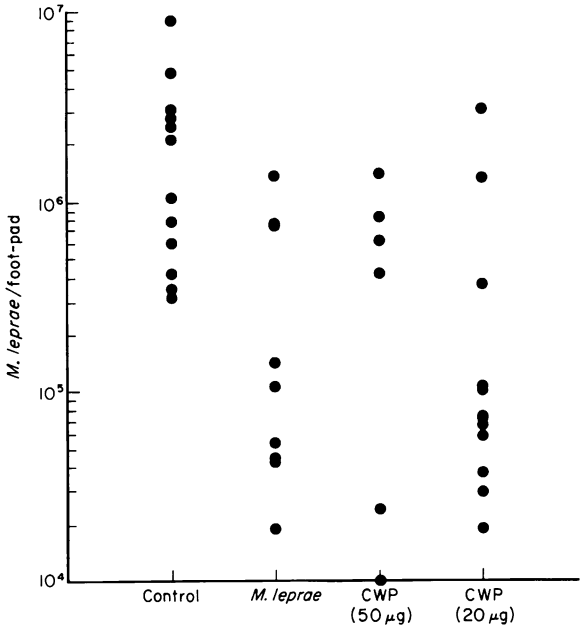

Fig. 2. Comparison of the growth of *M. leprae* in the foot-pads of mice infected with 10^4 *M. leprae* per foot-pad 1 month after subcutaneous vaccination with dead *M. leprae* (10^8 acid-fast bacilli), *M. leprae* CWP (50 μg), CWP (20 μg) or PBS emulsified in Freund's incomplete adjuvant and injected into both hind-flanks.

without leprosy exposure, presumably due to the presence of other cross-reactive mycobacterial proteins in the MLW 1 preparation [20]. Recent work on purified cell walls of *M. leprae* isolated proteins which stimulated T cell clones derived from the blood or skin lesion of a small number of PB leprosy patients and their contacts [10,11]. Preliminary characterization of the cell wall-derived proteins indicated they were unrelated to the antigens previously identified with monoclonal or polyclonal antisera [2]. Subsequently, SDS extraction of isolated *M. leprae* cell walls yielded a range of proteins including a dominant 17-kD species [12].

The strong cellular immune responses to the CWP in PB leprosy patients and contacts suggest that these proteins are significant in the host response to leprosy. Although this response was only present in half of the PB patients studied, those unresponsive to CWP were also unresponsive to either intact or sonicates of *M. leprae* (Table 1). A smaller proportion of PB patients responded to *M. leprae* than the 70% usually observed [21], even though all had intact cellular immune responses to mitogens (data not shown). This was due to the absence from the group of polar TT patients who have strong cellular immune responses to *M. leprae* [22]. There was a strong correlation between the proliferative responses to CWP and sonicates of *M. leprae* (Table 2). The relationship between CWP responsiveness and lepromin skin-test reactivity was less clear, as 45% of lepromin positive subjects were unreactive to CWP. Clearly there are additional antigens in lepromin capable of eliciting a granulomatous response in patients who do not respond to CWP. It is not known whether the responses to these proteins in PB leprosy patients are qualitatively different from those of contacts, particularly in the pattern of cytokine release by responding T cells. Such differences may be important in the outcome of infection, either eradication of the bacillus in control subjects or limited growth associated with damage to nerve and skin in PB patients.

The unresponsiveness of MB patients to the CWP mirrors the anergy observed to the whole bacillus at this pole of the disease. By contrast, cellular recognition of the *M. leprae* 70-, 65-, and 18-kD proteins occurs in some MB leprosy patients [8,9] (and unpublished observations) indicating that the cellular anergy in MB leprosy does not always extend to these antigens. This may be due to priming by cross-reactive homologues of the ubiquitous 65-kD and 70-kD proteins in other mycobacterial or non-mycobacterial organisms [21].

Self-limiting growth of *M. leprae* occurs in the foot-pad of the mouse [23]. Protection against *M. leprae* infection can be induced by prior vaccination with live or dead *M. leprae*, BCG [24] or *M. habana* [25]. Recently, immunization with cell wall preparations of *M. leprae*, including extractable cell wall proteins, resulted in significant inhibition of the growth of *M. leprae* and corresponding T cell reactivity [13]. Although the degree of protection induced by immunization with the CWP in the present study was variable (Fig. 2), the significant inhibition observed with 20 μg of CWP suggests that the preparation contains proteins capable of stimulating protective immunity.

While the relationship between proliferative responses in the peripheral blood and protective immunity remains uncertain and the mouse foot-pad is less than adequate as a model of leprosy disease, those proteins elicting strong responses in endemic exposed healthy controls and capable of preventing *M. leprae* growth in the mouse foot-pad must receive high priority for inclusion in potential leprosy vaccines.

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