A comparison of lymphoproliferative responses to *Mycobacterium leprae* in healthy contacts, tuberculoid and long-treated lepromatous leprosy patients

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Abstract

The lymphoproliferative responses to *Mycobacterium leprae* and BCG, for comparative purposes, were studied in three groups of individuals: 1) a group of 8 healthy contacts (HC) of leprosy patients, 2) a group of 3 tuberculoid leprosy (TT) patients and 3) a group of 3 long-treated lepromatous leprosy (TLL) patients. All subjects in the study responded positively to *M. leprae* with mean responses of 27565 cpm, 36489 cpm and 8312 cpm in the HC, TT and TLL groups respectively. The similar response patterns in the three groups to the 18 and 65 kDa antigens of *M. leprae* showed that these antigens could not be used to discriminate between patients and normals or between TT and LL patients. Analysis of the CD4/ CD8 percentages of the lymphoproliferative responses to *M. leprae* (62/28 in HC, 76/24 in TT & 51/18 in TLL) revealed that the HC, TT and TLL groups of individuals showed a similar CD4:CD8 ratio with the CD4 cell being the predominant responding cell. The $\alpha\beta$ / $\gamma\delta$ analysis of the lymphoproliferative responses to *M. leprae* revealed that the $\gamma\delta$ percentages were comparable in the three groups of individuals (19 % in HC, 13 % in TT & 19 % in TLL).

Key words: Lymphoproliferative responses; *M. leprae*; healthy controls; leprosy patients

Introduction

Leprosy is a chronic infectious disease of the skin and peripheral nerves caused by the obligate intracellular bacteria *Mycobacterium leprae*. The disease embraces a wide range of clinical manifestations which are determined largely by the host's immune response to the invading bacteria (Britton, 1993). In tuberculoid leprosy (TT) a strong cell mediated response limits the disease to one or two clearly defined, sparsely infiltrated lesions, while the absence of such a response in lepromatous leprosy (LL) results in undefined, heavily infiltrated, extensive lesions (Ridley & Jopling, 1966). Most individuals who come into contact with leprosy rarely develop the disease (Godal, 1978). These exposed individuals, generally, acquire the ability to respond to *M. leprae*. This ability can be measured *in vivo and in vitro* (Godal *et al.*, 1972, Godal & Negassi, 1973; Myrvang *et al.*, 1975). Thus, healthy contacts provide a unique opportunity to study an effective immune response to *M. leprae* and to compare compare it with responses in leprosy patients. A comparative study such as this may reveal differences which could prove useful in the development of a diagnostic test or a vaccine.

Lymphoproliferation, as measured by the in *vitro* lymphocyte transformation test (LTT) was used, first, to confirm that healthy contacts of leprosy patients develop a cellular response to *M. leprae*. The LTT was then used to compare the responses in three groups of individuals *ie.* 1) these healthy contacts of leprosy patients, 2) TT leprosy patients, and 3) long-treated lepromatous leprosy patients (TLL) who had shown a recovery in their ability to respond to *M. leprae in vitro* (Gill *et al.*, 1990). In this context, the LTT offers essentially, two advantages. First, it can be carried out repeatedly without affecting the response of the subject, unlike the lepromin test which is considered a microvaccination (Godal, 1978). Secondly, the LTT allowed us to examine and compare the antigens eliciting and the cells mediating the responses in these three groups of subjects.

Lymphoproliferative assays were used to examine the responses of the subjects to the 18 kDa and 65 kDa antigens of *M. leprae*. Similarly, the cells mediating the lymphoproliferative responses were analysed by flow cytometry with tricolour staining. Tricolour staining allowed us to determine the CD4/CD8 composition of a response by enumerating cells that carried the HLA-DR antigen, the CD3 antigen and either the CD4 or the CD8 antigen. Similarly, the determination of the $\alpha\beta/\gamma\delta$ composition of a response involved the enumeration of cells that carried the HLA-DR antigen, the CD3 antigen, and either the $\alpha\beta$ or the $\gamma\delta$ antigen. The HLA-DR marker, which is a marker for activation (Cotner et al., 1983; Crabtree, 1989) was used to ensure that only participating

cells were included in our calculations.

In this study, healthy contacts of leprosy patients were screened for responsiveness to *M. leprae* in proliferative assays. All these contacts were found to respond to *M. leprae*. The lymphoproliferative responses in these healthy contacts were compared with those in TT and TLL patients to determine what constitutes a protective antigen and a protective response in leprosy.

Materials and Methods

Subjects

The subjects studied comprised three groups of individuals. The first group (N = 8) was made up of healthy family contacts and occupational contacts of leprosy patients. The two occupational contacts, AAM and HKG, had worked with leprosy patients for 19 and 15 years, respectively. In addition, AAM (Smelt *et al.*, 1981) and HKG (Gill *et al.*, 1986) had been vaccinated with the heat-killed, armadillo derived *M.leprae* vaccine. Three more healthy, family contacts were included in the screening for responses to the 18 and 65 kDa *M. leprae* antigens. The second group of individuals consisted of tuberculoid leprosy patients (N = 3). The third group of individuals consisted of lepromatous leprosy patients (N = 3) who had been treated for more than 20 years and who had been found to respond to *M. leprae* in the LTT. There were five TLL patients who responded to *M. leprae* in that study but only three of them were available for this study.

Lymphocyte Transformation Test

Peripheral blood from each subject was collected in preservative-free heparin. The mononuclear cells were isolated from whole blood by density centrifugation on a Ficoll-metrizoate gradient (Lymphoprep; Nyegaard & Co., Oslo, Norway). The peripheral blood mononuclear cells (PBMC) were cultured at a concentration of 10^5 cells/well in 96-well U-bottom trays with antigen added in triplicate. The trays were incubated 6 days at 37°C in a humidified atmosphere of 5 % CO_2 and 95 % air. On day 6, the culture was pulsed with 0.045 Mbq ^{3}H-thymidine (specific activity = 185 x 10^3 Mbq/mmole) for 4 hours, after which they were harvested with a Skatron Harvestor (Norway). The radioactivity incorporated was determined by liquid scintillation spectroscopy. The results were expressed as the mean value of counts per minute of triplicate cultures. An individual was considered a responder to a given antigen when T/C = (cpm of lymphocytes + antigen) /(cpm of lymphocytes) >2 and Δ cpm = (cpm of lymphocytes + antigen) - (cpm of lymphocytes) >1000.

Antigens

The *M. leprae* antigens were kindly provided by Dr RJW Rees (Mill Hill, London). Two soluble, whole *M. leprae* antigenic preparations, CD 132 and CD 165 were used in this study. Whilst the two preparations were not identical, both consistently elicited positive responses in the same individual. BCG and PPD were obtained from the Serum Institute, Copenhagen, Denmark. The *M. leprae* & BCG antigens were used at concentrations of 10 µg/ml and 1 µg/ml in culture. The 18 kDa and the 65 kDa antigens were kindly provided by the Steering Committee of Immunology of Leprosy Scientific Working Group (IMMLEP) of the UNDP/World Bank/ WHO Special Programme for Research and Training in Tropical Diseases (TDR). These antigens were used at 2.5 µg/ml in culture.

Cytofluorometric Analysis

PBMC cultured as for an LTT, were collected and placed in a tube (Falcon 2054, Becton Dickinson). The cells were spun at 300 G for 10 minutes and resuspended in an isotonic solution (Haemaline-2, Serono Baker Diagnostics, Allentown, PA). 10 µl of the appropriate monoclonal antibody was added to 100 ul of the cell suspension containing approximately 1 x 10^5 cells. The mixture was incubated for 30 minutes, after which 2 ml of isotonic diluent was added to each tube of cells. The tubes were centrifuged for 5 minutes at 300 G. The supernatants were discarded and 0.4 ml of isotonic diluent was added to each tube. The cells were then resuspended and stored at 4°C in the dark before analysis. The cells were analysed within 4 - 6 hours of staining.

The monoclonal antibodies used were : anti-TCR αβ FITC (Cat. no. 347773), anti-TCR γδ FITC (Cat. no. 347903), anti-CD4 FITC (Cat. no. 347323), anti-CD8 FITC (Cat. no. 347313), anti-HLA-DR PE (Cat. no. 347367) and anti-CD3 PerCP (Cat. no. 347344) (all monoclonals used were from Becton Dickinson).

Tri-colour analysis was carried out using the flowcytometer (FACScan, Becton Dickison, Mountain View, CA) and Lysys II Software. Computer files were then transferred using HP-Reader Version 2 and the analysis of the data was carried out using Pc-Lysys Software Version I (Becton Dickinson). Three regions were set, 1) for resting & activated lymphocyte population (R1), 2) CD3 positive cells (R2), and 3) HLA-DR positive cells (R3). Only cells that were positive for all three criteria were analysed for their αβ/γδ and CD4/CD8 staining.

Results

Responses to soluble whole M. leprae antigen and BCG in healthy contacts, TT patients and TLL patients

All eight contacts (Table 1) responded positively to the soluble whole *M. leprae* antigen preparation with a mean proliferative response of 27565 cpm. The three subjects in the TT group also responded positively to *M. leprae* with a mean proliferative response of 36489 cpm. Although the mean response to *M. leprae* in the TLL group was 8312, all three individuals responded positively to this antigen. All the individuals in the study responded positively to BCG, with mean responses of 37106 cpm, 21205 cpm and 24591 in the contact, TT and TLL groups respectively.

Proliferative responses to the 18 kDa and 65 kDa M. leprae antigens in healthy contacts, TT patients and TLL patients

The response patterns to the 18 kDa and 65 kDa *M. leprae* antigen were more heterogeneous (Table 2). The following patterns 1) a positive response to the 18 kDa antigen only, 2) a positive response to the 65 kDa antigen only and 3) positive responses to both the 18 and 65 kDa antigens, were observed in the responses of all the subjects in the TT and TLL groups, and in some of the subjects of the contact group. A fourth pattern ie. no response to both the 18 and 65 kDa antigens, was also observed in four subjects in the contact group.

Table 1. Proliferative responses to *M. leprae* and BCG in healthy contacts, TT patients and TLL patients.

	Control	BCG	CD 132
Contacts			
AAM	2450	27883	37019
HKG	2239	34475	75730
AMAR	2511	46295	12730
RSAR	2611	46560	35137
AM	2135	29698	21644
SMZ	591	15469	10167
MAMZ	2932	27740	15308
KMZ	732	31622	12787
TT			
EKC	1812	19113	24692
YT	6462	17116	23027
YA	4053	27386	61747
TLL			
LLH	6023	33357	16763*
SSM	837	11395	3632
TLY	1934	29020	4514

CD 132 is a whole *M. leprae* soluble antigen preparation. * is a response to CD 165, a different batch of soluble *M. leprae*. The results are expressed in mean cpm of triplicate cultures.

Table 2. Proliferative responses to the 18 kDa and 65 kDa *M.leprae* antigens in healthy contacts, TT patients and TLL patients.

	Control	BCG	CD132	CD165	18 kDa	65kDa
Contacts						
AAM	1183	32250	19123		1228	4816
HKG	1836	23964	44889		11708	12970
AMAR	662	10371		6330	1364	2152
RSAR	6422	23816		14686	791	1907
AM	1897	14903		11695	2464	4379
SMZ	537	11408		11873	643	622
MAMZ	947	17041		4585	383	1408
KMZ	3787	14151		11161	713	1509
RMZ	1341	16888		10559	8963	5721
YAH	1516	19020		21349	7467	5945
NM	970	14743		14157	3141	915
TT						
EKC	1781	28502		15016	4562	3741
YT	5543	32874		17217	13070	6975
YA	1571	10082		88577	2569	3364
TLL						
LLH	1491	36272		ND	4650	6396
SSM	2810	41311	22719	22319	14547	4859
TLY	1243	26575	3830		2617	1375

CD 132 and CD 165 are whole M. leprae soluble antigen preparations. The results are expressed in mean cpm of triplicate cultures. Positive responses to the 18 kDa and 65 kDa antigens are underlined.
ND : Not done

The CD4/CD8 profile of the responses to M. leprae and BCG in healthy contacts, TT patients and TLL patients

The CD4 and CD8 percentages, expressed as an average percentage of the CD3+, HLA-DR+ response to *M. leprae* (CD 132 and CD165), were 62/28, 76/24 and 51/18 in the contact, TT and TLL groups, respectively (Fig. 1). Whilst the range of the CD4/CD8 percentages of the response to *M. leprae* was within the range of the CD4/CD8 percentages observed in the two other groups, the average CD4 and CD8 percentages seen for this antigen were lower in the TLL group. The CD4/CD8 percentages, expressed as an average percentage of the CD3+, HLA-DR+ response to BCG, were 74/22, 76/15 and 83/9 in the contact, TT and TLL groups, respectively. Again the average CD4/CD8 pecentages in the TLL group were inconsistent in that the average CD4 percentage of the response was higher and the average CD8 percentage of the response was lower. However, the range of the CD4/CD8 percentages fell within that seen in the contact and TT groups.

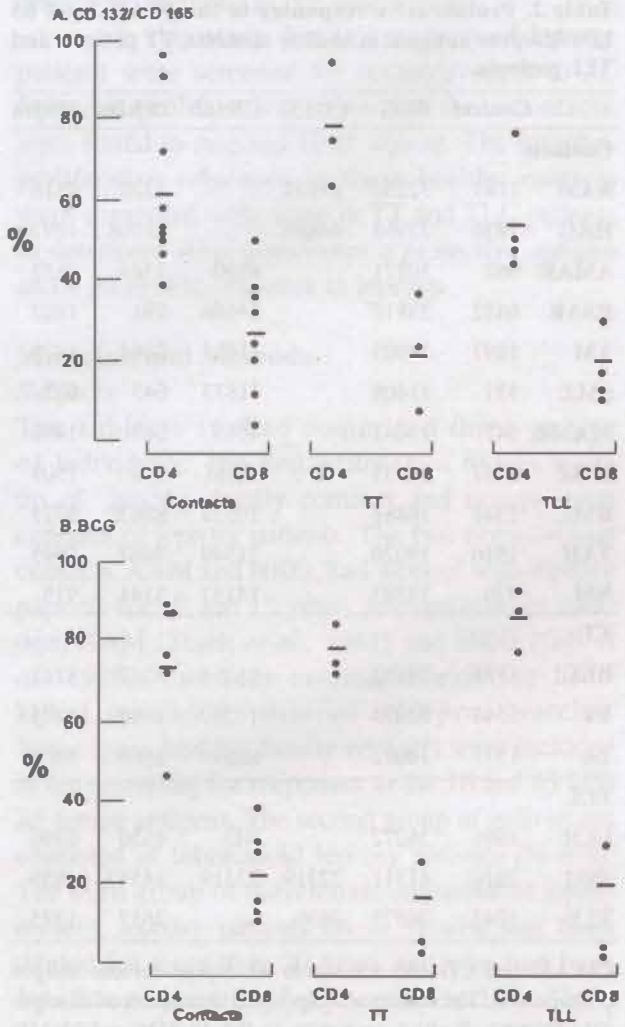

Fig. 1. CD4/CD8 percentages of CD3+, HLA-DR+ responses to M. leprae (CD132/CD165) and BCG in Healthy Contacts, TT and TLL patients. Each dot represents the CD4 or CD8 percentage of the responding cells of one subject. Occasionally, one subject's responses to both CD132 and CD165 may be depicted in the figure. Horizontal bars represent the mean percentage for the group.

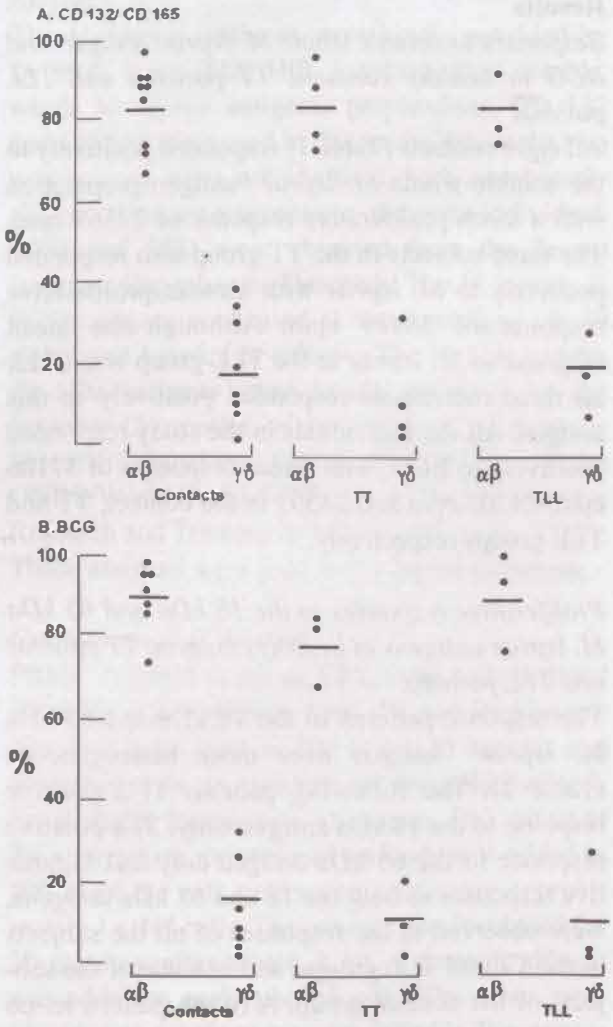

Fig. 2. αβ/γδ percentages of CD3+, HLA-DR+ responses to M. leprae (CD132/CD165) and BCG in Healthy Contacts, TT and TLL patients. Each dot represents the αβ or γδ percentage of the responding cells of one subject. Occasionally, one subject's responses to both CD132 and CD165 may be depicted in the figure. Horizontal bars represent the mean percentage for the group.

The αβ and γδ profile of the responses to M. leprae and BCG in healthy contacts, TT patients and TLL patients

The average αβ/γδ percentages of the CD3+, HLA-DR+ response to *M. leprae* were 84/19, 84/13 and 84/19 in the contact, TT and TLL groups, respectively (Fig. 2). All three groups displayed similar ranges in the αβ/γδ percentages of their responses to *M. leprae*. The average αβ/γδ percentages, of the CD3+, HLA-DR+ response to BCG, were 89/13, 79/11 and 89/11 in the contact, TT and TLL groups, respectively. The range of the αβ/γδ percentages of the responses to BCG were similar in the three groups of subjects.

Discussion

It is well documented that the contacts of leprosy patients generally develop a cellular response to *M. leprae* Since only family contacts who were 15 years and older were included in this study, the positive lymphoproliferative responses seen in all these individuals is not unexpected. The two occupational contacts who had both 1) worked with leprosy patients for 19 and 15 years, and 2) had been vaccinated with the killed, armadillo-derived *M. leprae* vaccine, also responded postively to *M. leprae* in vitro. Having confirmed that all the contacts responded to *M. leprae* in vitro the choice of the parameters examined in this, essentially quantitative, comparison of the lymphoproliferative responses in a small study population of healthy contacts, TT patients and TLL patients was, to some extent, determined by the availability

of the TLL group.

The lymphoproliferative responses, in the three groups of subjects, to the 18 kDa *M. leprae* antigen were studied mainly because this was the antigen that elicited a response from a panel of T cell clones raised from volunteers who had participated in a phase I trial of the killed, armadillo-derived *M. leprae* vaccine (Mustafa *et al.*, 1986). The 65 kDa was included in the study for comparative purposes. Both these antigens are heat shock or stress proteins (Young *et al.*, 1992). Our study revealed that the three groups of subjects responded to the 18 kDa and 65 kDa antigens in much the same way. Both antigens could not be used to discriminate between healthy contacts and patients or between TT and LL patients. This is in agreement with the study by Thole et al. (1995) in which they examined the in vitro proliferative responses of healthy controls, Tuberculoid/ Borderline Tuberculoid (TT/BT) patients and LL patients to a panel of 11 *M. leprae* antigens, which included the 18 and 65 kDa. antigens. They found that there was no association between specific responses and subject status. An interesting finding of this study was that 15 % of the patients who were non-responsive to sonicates of *M. leprae* showed significant T cell responses to one or more individual antigens. Thole et al. (1995) noted that since these antigens could not be responsible for the *M. leprae* specific non-responsiveness seen in LL patients, they should be included in a vaccine against leprosy.

The study of the CD4/CD8 percentages of the proliferating cells, from subjects in the three groups, was prompted by reports of the isolation of CD8 cells with suppressor functions from BL and LL patients (Ottenhoff *et al.*, 1986; Modlin *et al.*, 1986; Salgame *et al.*, 1991). The results of our study, however, revealed that the CD4/CD8 percentages in the response to *M. leprae* showed a similar ratio in the HC (2:1), the TT (3:1) and the TLL (2.8:1) with the CD4 cell being the predominant responding cell. Yet it must be noted that the CD4/CD8 percentages, in the response to *M. leprae* of the TLL group (51/18), were lower than those seen in the two other groups (62/28 in HC & 76/24 in TT). Furthermore, the sum of the two percentages in the TLL group was below the expected value of 100. This may be explained by the possibility that the responding cell population included a group that was both CD4- and CD8-.

Reports that γδ T cells play a role in the defence against mycobacterial diseases (Modlin *et al.*, 1989; Uyemura *et al.*, 1991; Barnes *et al.*, 1992) and the possibility that γδ T cells may account for the missing component in the CD4/CD8 analysis as they are CD4-CD8- or CD4-CD8+ dimly positive, lead to the examination of the αβ/γδ percentages in the proliferating cells from our subjects. It must be noted here that we were still using the same strategy of enumerating these subsets in a population of cells that were both CD3+ and HLA-DR+. We observed that the αβ/γδ components of a response generally add up to 100, as conceivably there are no CD3+ T cells without a T cell receptor. This does lend support to the possibility considered above, that the CD4/CD8 analysis includes a population of participating cells that are CD4- and CD8-. Our results revealed that the average percentages of αβ/γδ cells were 84/19, 84/13 and 84/19 in the healthy contacts, TT patients and TLL patients respectively. That is to say, that the average γδ component of the proliferating cells in the TLL group was not unusually large or different from that of the other two groups. In their study on the involvement of γδ T cells in the response to mycobacteria, Barnes *et al.* (1992) reported that after culture in vitro with *Mycobacterium tuberculosis* (either heat-killed H37Ra or live H37Ra) and further expansion of the proliferating cells with interleukin-2 (IL-2), the γδ percentage of the response was 25 % in tuberculin reactors, 30 % in patients with tuberculous pleuritis, 9 % in patients with advanced pulmonary tuberculosis, and 2 % in miliary tuberculosis. They also showed that in the proliferative responses of leprosy patients to *M. tuberculosis*, the γδ percentage was 32 % in TT patients, and 9 % in unresponsive LL patients. The authors concluded that the percentages of γδ cells were significantly higher in patients with protective and resistant immunity than in those with ineffective immunity. There are major methodological differences between the Barnes *et al.* (1992) study and our study. Firstly, their strategy to enumerate specifically responding cells was to culture the peripheral blood mononuclear cells with H37Ra for five days and then to expand the blast cells from the primary culture by further culture with IL-2. This procedure would account for the higher percentages seen in their study. Secondly, because of the difference in strategy, they used just two markers ie. CD3 and either αβ or γδ to identify and enumerate the relevant subsets. We, on the other hand, used the HLA-DR marker to ensure that we were enumerating only responding cells. Both Haregewoin *et al.* (1989), who established a γδ T cell line from a BCG-immune individual and Langhorne *et al.* (1992) who reviewed the role of γδ T cells in the proliferative responses of non-exposed individuals to *Plasmodium falciparum*-infected erythrocytes or merozoites, showed that their γδ T cells expressed

cell-surface HLA-DR activation antigens. The third difference involves using *M. tuberculosis* to study the γδ T cell response in leprosy patients. As we were studying an unusual group of individuals LL individuals who had recovered, partially, their ability to respond to *M. leprae in vitro*, we were able to use the specific antigen in our study. The fourth difference lies in the use of live versus dead or sonicated antigen. The Barnes et al. (1992) study used both dead and live antigen, while we used sonicated whole antigen. It has been shown by Havlir et al. (1991) that live organisms are better at eliciting a γδ response.

The general consensus, based on studies with knock-out mice, is that immunity to intracellular organisms such as Mycobacteria, involves both CD4 and CD8 cells (Ladel *et al.*, 1995; Maw *et al.*, 1995; Kaufmann, 1995). The relative importance of the CD4 cell was elucidated in immunohistopathological studies which revealed that while tuberculoid granulomas present an organised picture with a central aggregation of epitheloid cells interspersed with CD4 T cells surrounded by a mantle of CD8 cells, lepromatous granulomas lack organisation and contain very few CD4 and CD8 cells randomly distributed among the parasitized macrophages (Modlin *et al.*, 1983). The ratio of CD4/CD8 cells ranges between 2 and 5 in tuberculoid granulomas and falls below 1 in lepromatous granulomas (Narayanan *et al.*, 1983). The change in ratio is a reflection of the changing numbers of CD4 T cells in the two lesions. Our study shows that while TLL patients respond to *M. leprae*, the proliferative responses, on the average, are low. This may be due to a lower number of CD4 and, to a lesser extent, CD8 *M. leprae* reactive T cells or to the abrogation of their functional capabilities (Britton, 1993), such as the production of IL-2. Bearing in mind that 19 % of a 8312 cpm response is considerably less than 19 % of a 27565 cpm response, it is important to note that the reasonable γδ response in the TLL individuals does not compensate for a lack of antigen reactive and functional CD4 cells. This is in general agreement with the view expressed by Haas *et al.* (1993) that γδ T cells complement the central role played by αβ T cells in the defence against microbial infections.

The studies towards this end have made some progress in answering the question of what constitutes protective antigens and a protective response in leprosy. However, we agree with Thole *et al.* (1995) in that it may be more informative, in future studies, to examine an effector function of protective imuunity such as cytokine production or cytolysis and to use the antigens that will be become available from the *M. leprae* genome project (Cole, 1994; Phillip *et al.*, 1996).

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