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Original Article

Study of cytokine response against panel of purified *Mycobacterium leprae* antigens by using whole blood assay in subjects residing in a resettlement village of cured leprosy patients

RR Kamble, VS Shinde, SP Madhale, RS Jadhav

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Mycobacterium leprae being an intracellular pathogen, cell mediated immunity is very important in the clinical outcome of leprosy. Manifestation of the disease is correlated with the level and type of cell mediated immune response. The main objective of this study was to analyse TNF-α and IFN-γ production by T-cells when challenged with different *M. leprae* purified antigens in subjects with known exposure. 50 subjects residing in resettlement village of cured leprosy patients were included in the study. Whole Blood assay studies were undertaken in which the blood was placed in culture and was challenged with 35-kDa antigen, whole *M. leprae* cells, *M. leprae* cell wall antigen and *M. leprae* soluble antigen minus LAM. T-cell derived cytokines TNF-α and IFN-γ were measured by ELISA. It was observed that challenging the lymphocytes with 35-kDa antigen, the cell wall antigen and *M. leprae* soluble antigen minus LAM resulted in increased levels of IFN-γ whereas challenge with 35-kDa antigen and *M. leprae* cell wall antigen resulted in increased levels of TNF-α.

Keywords : Cytokine response, PCR, Contacts, Leprosy

Introduction

Despite success of MDT in reducing prevalence of leprosy since its implementation in 1982, the detection rate of new cases has not shown a similar decline. The global leprosy situation at beginning of 2007 was around 2, 31,361 cases as registered prevalence and the new cases detected in 2006 were 2, 65,661 (Weekly Epiemiological Record 2007). In endemic areas, untreated lepromatous case may be a potent source of infection in the community as they shed large number of bacilli in the environment through nasal secretions or ulcerated nodules on the skin. There are many reports which suggest that the risk of acquiring leprosy is greater among the contacts of MB patients than the non contacts (van Beers et al 1999). The WHO strategy for global elimination of the disease depends on early detection of leprosy and effective treatment with MDT. Although the household contacts are important in the transmission, it has been reported that 70% of new cases have no

RR Kamble, MSc, Research Fellow
VS Shinde, MSc, Research Fellow
SP Madhale, MSc, Research Fellow
RS Jadhav, PhD, Scientist In-charge
Stanley Browne Laboratory
TLM Community Hospital, Nandnagri, Shahdara, Delhi-110 093, India
Correspondence to : RS Jadhav **Email :** rupenjadhav@yahoo.com

confirmed history of leprosy contact (Smith and Smith 2000). There is increasing evidence from nasal polymerase chain reaction studies that sub-clinical transmission may exist and that mucosal immune responses to *M. leprae* may develop during resolution of initial infection (Ramaprasad et al 1997). Sub-clinical infection may confound control strategies; therefore, use of screening tests to identify asymptomatic highly infectious cases for earlier therapy is required (Baumgart et al 1993). Serological assays based on *M. leprae* specific antigens mainly phenolic glycolipid 1 (PGL1) has been used to monitor *M. leprae* infections as antibody levels are correlated with intensity of exposure and systemic involvement of *M. leprae* (Convit et al 1983). However, paucibacillary patients do not develop significant antibody levels, therefore, the applicability of serological tests is limited in such conditions. PCR based amplification of *M. leprae* DNA has been used for the detection of *M. leprae* as the technique is sensitive, specific and rapid. PCR technique could be of great importance in studying leprosy transmission (Smith et al 2004) and sub-clinical infections in endemic population (Job et al 1997). But, as of date the significance of such detection in relation to the process of infection is not yet established.

Measurement of cell-mediated immunity is probably the most relevant in leprosy, as *M. leprae* is an intracellular pathogen. *M. leprae* specific antigens can make such assays useful to measure exposure of *M. leprae* within communities or populations or to aid the early diagnosis of leprosy (Dockrell et al 2000). Using comparative genomics, candidate proteins highly restricted to *M. leprae* showed promising features with respect to application in leprosy diagnostics (Geluk et al 2008). The whole blood assays (WBA) provide reproducible *in vitro* measurements that can be used to monitor changes in T-cell responses to *M. leprae* antigens (Weir et al 1998). In the present study, we analyzed TNF-α and IFN-γ production by T-cells when challenged with different *M. leprae* purified antigens like 35-kDa, cell wall antigen (MLCwA), *M.leprae* soluble antigen minus LAM (MLSA-LAM) and whole *M. leprae* cells (WML) in subjects with known exposure and or history of exposure.

Materials and Methods

50 subjects residing in resettlement village of cured leprosy patients situated near Richardson Leprosy hospital, Miraj, Maharashtra were included in the study. Of these, 13 were old cured multibacillary patients, 10 paucibacillary and 27 other individuals (healthy household contacts and individuals residing in the village). Nasal swabs were collected and immediately chilled and transported to the Laboratory for the detection of *M. leprae* by PCR. 5 ml Blood samples were collected in a falcon tube containing 100 µl of 1000 units/ml heparin.

Antigens and *M. leprae* DNA

Cell wall antigen (MLCwA), *M.leprae* soluble antigen minus LAM (MLSA-LAM) and whole *M. leprae* cells (WML) used in the WBA and *M. leprae* DNA were kindly provided by Prof Patrick Brennan from Colorado State University. 35 kDa antigen was obtained from Anandban Mycobacterial Research Laboratory, Nepal (original source Prof Warwick Britton, Australia).

Detection of *M. leprae* nasal carriage

Subjects were tested for nasal carriage of *M. leprae* by PCR as described earlier (Jadhav et al 2001); briefly, swabs (Medical Wire and Equipment, UK), dipped in sterile saline immediately prior to use, were passed along the base of the inferior turbinate until the posterior wall of the naso-pharynx was encountered: one swab was taken from each nostril. Swabs were immediately chilled and transported to the laboratory. The end of the swab was cut off, immersed in 150 µl of lysis buffer (1mg/ml Proteinase K in 100 mM Tris-HCl, pH 8.5, 0.05% Tween 20) and incubated under mineral oil (Sigma, UK) at 60°C overnight. Proteinase K was then inactivated by incubation at 97°C for 15 minutes. The vials were centrifuged for 10 s at

10,000 x g, to ensure that the cotton wool remains in the lysis buffer. 2 µl of the lysate was subsequently used for PCR. The primers used were S13 5'-CTCCACCTGGACCGCGAT-3' and S62 5'-Bio-GACTAGCCTGCCAAGTCG-3' which amplify a 531 bp fragment of *M. leprae* proline-rich antigen (*pra)* gene. Amplification was carried out with the following conditions; initial denaturation at 95°C for 5 min followed by 37 cycles consisting of denaturation at 94°C for 2 min, annealing at 55°C for 2 min and extension 72°C for 3 min. Amplified product was detected by an ELISA based assay and positive samples were confirmed by Southern blot technique as described earlier (Jadhav et al 2001). In brief, for an ELISA and Southern blot, 10 µl from each 25 µl PCR reaction was mixed with 2 Pmol of a fluorescein-labeled PNA oligonucleotide probe (Perseptive Biosystems, UK) internal to the *M. leprae pra* PCR product (5'- Fluo-CCCAGCCACGGTCCT-3') in a final volume of 40 µl, heat denatured at 95°C for 10 min, and allowed to cool slowly to room temperature over 2 hours. The product was either captured on the streptavidin coated plate for the ELISA or run on the gel and then transferred on the nylon membrane. Detection of the hybridised product was carried out using anti-fluorescein antibody tagged with horseradish proxidase. For an ELISA, the DNA:PNA hybrid was added to these plates, incubated at room temperature (27°C+/-1°C) for 90 min and washed at room temperature with Tris-buffered saline with 0.1% Tween 20 (TBS/T) 5-7 times over a period of 30 min. Plates were blocked with 200 µl of 2% BSA in TBS/T for 90 min at room temperature and washed as previously. 50 µl of horseradish peroxidase-conjugated (HRPO) anti-fluorescein antibody (Sigma, UK, diluted 1:2000 in TBS/T) was added at room temperature for 90 min, the plates again washed as described and 50 µl of substrate added (1mg/ml O-phenylenediamine (OPD) in Phosphate-Citrate buffer with 0.1% H_2O_2; Sigma, UK). The reaction was terminated after 15 min incubation in the dark at room temperature by addition of 50 µl 3 M HCl and the optical density read at 490 nm on a Dynatech MR 5000 ELISA reader.

For Southern blot, hybridised samples were loaded on 2% agarose gel, run at 75 V for approximately 2 hours and immediately transferred onto positively charged nylon membrane (Boehringer Mannheim, UK) by capillary transfer; standard southern transfer denaturation and neutralisation steps were not undertaken as the samples were hybridised prior to electrophoresis. Membranes were blocked overnight with constant agitation at room temperature in TBS/T with 2% BSA, incubated in 25ml Alkaline phosphatase-conjugated anti-fluorescein antibody (1:2500 in blocking solution) for 2 hours at room temperature with constant agitation for detection and washed 6-7 times with TBS/T over 40 min to remove excess antibody. Colour development was by incubation for 10-15 min at room temperature in 10 ml developing solution (0.38 mM BCIP (5-Bromo-4-chloro-3-indolyl phosphate) and 0.41 mM NBT (nitroblue tetrazolium) in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl2; pH 9.5) Development was terminated by addition of EDTA in PBS to a final concentration of 0.2 mm.

All negative samples were tested using primers for human β-globin gene which gives 212 bp amplicon as an internal positive control to ensure that the PCR reaction had worked (PC04: 5'-CAACTTCATCCACGTTCACC-3' and GH20: 5'-GAAGAGCCAAGGACAGGTAC-3'). PCR conditions were same as decribed above. Positive control reactions contained 250 pg of purified *M. leprae* DNA. PCR positive samples were amplified with another set of *M. leprae* specific primers (RLEP) to cross check the results obtained with *pra* primers. The RLEP primers (PS3 : 5'-GGACACGATTAGCGCGCACGT-3' and PS4 : 5'-Bio-TTGTGGTGGGCTGGTGGGGTGTGG-3') (Jamil et al 1994) were used at concentration of 50 ng each per reaction. Reaction conditions were: initial denaturation 95°C, 5 min; 37 cycles of : 94°C,

1 min; 65°C, 1 min; 72°C, 2 min; and one cycle of 72° C for 10 min. Primer PS4 was biotinylated at 5'end. This generated a PCR product of 455 bp amplification product.

Whole blood assay

T cell responses to *M. leprae* specific antigens were measured with whole blood assay as described earlier (Weir et al 1998); briefly, heparinised blood sample was diluted in RPMI tissue culture medium containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and 2 mM L-glutamine (Gibco-BRL, Paisley, United Kingdom) to give a final dilution of 1 in 10. 200 μl of diluted blood was added in triplicate in wells of Falcon U bottomed plate (96 well plate). *M. leprae* antigens to be tested were added in the wells at 10 μg ml final concentration. Phytohaemaglutinin (PHA) was used at a final concentration of 5 μg/ml. Plates were incubated at 37°C with 5 % CO_2 for 5 days. Supernatants was harvested on day 6 and stored at -70°C prior to ELISA. PHA and plain RPMI were used as a positive control and negative control respectively.

Cytokine ELISA

Detection and quantitation of cytokines (TNF-α and IFN-γ) was done by using BD OptEIA set for ELISA (BD Biosciences Pharmingen) as per the instructions. The purified anti-cytokine capture antibody was diluted to 3 μg/ml in coating buffer. 100 μl of diluted antibody was added to the wells of an ELISA plate. The plate was sealed to prevent evaporation and incubated overnight at 4°C. The plate was then allowed to normalize at room temperature (RT), the capture antibody solution was removed and nonspecific binding was blocked by adding 200 μl of assay diluent solution per well. The plates were sealed and incubated at RT for 1 hour. After washing the plate three times with assay diluent, 100 μl of samples were added to the wells. Standards ranging from 300 pg/ml to 4.7 pg/ml were also used. Plates were incubated for 2 hours and then washed with assay diluent five times. After the wash 100 μl of biotinylated anti-cytokine detection antibody (0.5 μg/ml) was added to each well. Plates were incubated at RT for 1 hour. After washing 100 μl of 1:10,000 diluted avidin-horseradish peroxidase (Av-HRP) was added to each well. Plates were incubated at RT for 30 min. After washing, 100 μl/well of o-Phenylene diamine dihydrochloride substrate diluted 1 mg/ml in 10 ml phosphate citrate buffer containing 10 μl H_2O_2 (0.3% final concentration) was added and the plate was incubated in dark at RT for 15 min. The reaction was terminated by adding 50 μl/well 3N HCl. The optical density (OD) was measured using ELISA Reader (Dynatech MR 500) at 490 nm as test filter and 630 nm reference filter.

Statistical analysis

Data for the cytokine production in different sub-groups was analysed by using Student's t test for the the unequal sample sizes with unequal variance.

Results

PCR based detection of *M. leprae* DNA on the nasal swabs was carried out using primers specific for *pra* gene (Figure 1). PCR positivity was confirmed by retesting the samples with RLEP primers. Primers for β globin gene were used as internal controls and PCR reactions using samples with spiked *M. leprae* DNA were carried out to check for any PCR inhibitors. None of the samples showed any evidence of PCR inhibitor ruling out any false negative results. Negative controls with nuclease free water instead of sample were used to check any cross contamination or false positivity. To check and ensure appropriate amplification, the PCR products were tested by ELISA and Southern hybridisation (data not shown).

PCR results on the nasal swabs were positive for 33 subjects out of 50 (Table 1). Out of 13 cured MB patients tested, 7 subjects showed nasal PCR positivity whereas 8 out of 10 cured PB patients were positive for nasal PCR. Amongst 27 healthy subjects, 18 showed presence of *M. leprae* on the nasal swab suggesting a recent exposure (Table 1).

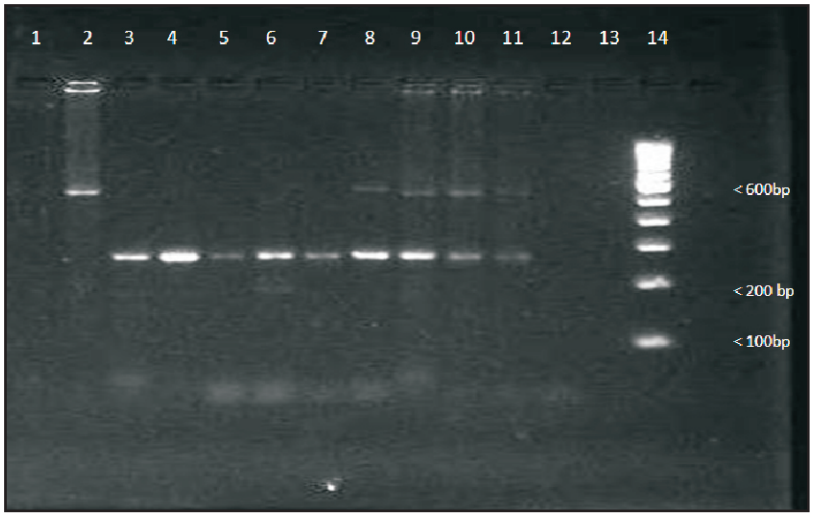

Figure 1 : Agarose gel electrophoresis showing PCR product of *pra* gene region (531 bp size) and β globin gene region (212 bp size). Lane 1 and 13: Blank, Lane 2: Positive control (*M.leprae* DNA). Lane 3-7: nasal swab lysates (showing only 212 bp product- β globin gene amplification). Lane 8-11: nasal swab lysates (showing both 212 bp- β globin gene and 531 bp *pra* gene amplicons). Lane 12: negative control. Lane 14: Molecular weight markers (100 bp ladder).

Table 1: PCR results of the nasal swabs collected from subjects

Subjects	PCR Positive	PCR Negative
Healthy contacts (27)	18	9
Cured MB patients (13)	7	6
Cured PB patients (10)	8	2
Total	33	17

Blood samples from these subjects were used in whole blood assay (WBA) to check the cytokine responses when challenged with different *M. leprae* antigens. The results of the stimulation of lymphocytes for the production of IFN-γ and TNF-α were analysed. When the lymphocytes were challenged with 35 KDa antigen, MLSA-LAM and the MLCwA the IFN-γ production was increased in all the subject groups (Figure 2a). For MLCwA, old PB patients and healthy contacts showed significantly higher response compared to old MB cases (p<0.05 and p<0.01 respectively). Overall response to WML was relatively low but within the subgroups the response in old MB cases was significantly higher compared to healthy contacts (p<0.05) and old PB cases (p<0.01). Response to 35 kDa antigen was highest in healthy contacts compared to both old MB and PB cases but not reaching statistical significance. MLSA-LAM showed overall increase in IFN-γ production in all subjects.

As shown in Figure 2b, TNF-α level increased when challenged with 35 kDa antigen and MLCwA. TNF-α level in response to 35 kDa antigen were significantly higher in healthy individuals compared to old MB cases (p<0.005) and old PB cases (p<0.00005). Similarly, TNF-α response to MLCwA antigen was significantly higher in healthy contacts compared to old MB cases (p<0.001) and old PB cases (p<0.0005). Though response to MLSA-LAM and WML was lower, healthy contacts showed highest response. Response to MLSA-LAM in healthy contacts was significantly higher compared to old MB cases (p<0.05) and old PB cases (p<0.005).

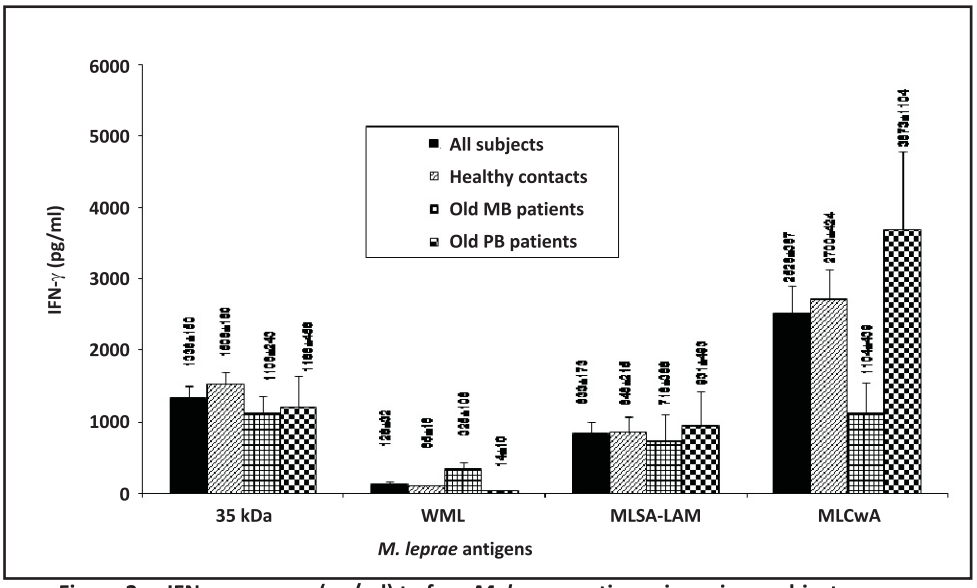

Figure 2a : IFN-γ response (pg/ml) to four *M. leprae* antigens in various subject groups.

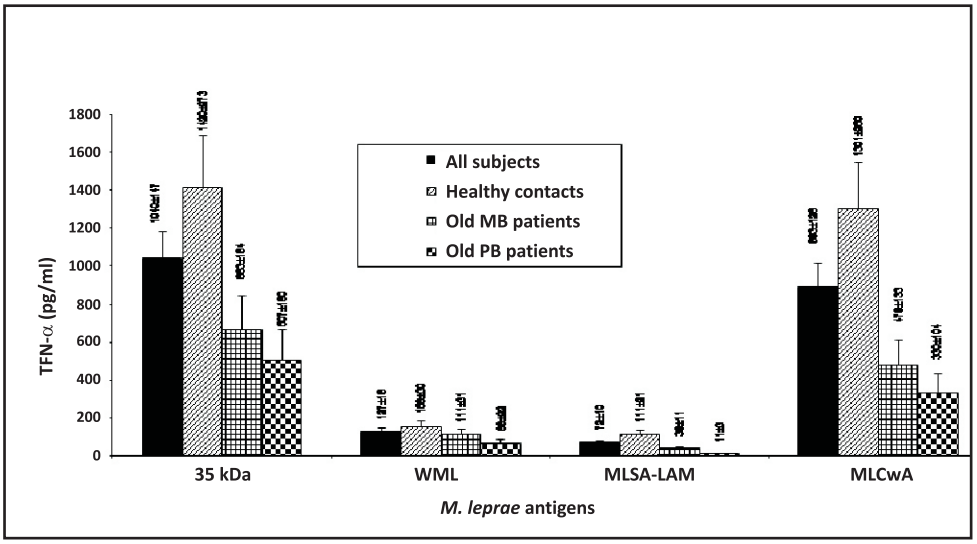

Figure 2b : TNF-α (pg/ml) response to four *M. leprae* antigens in various subject groups.

Cytokine response when analysed in response to PCR status reveal that the IFN-γ response was significantly elevated in PCR positive group than the PCR negative group when challenged with MLSA-LAM ($p<0.0005$) and MLCwA ($p<0.05$) (Table 2). Overall TNF-α response to 35 kDa and MLCwA was higher compared to WML and MLSA-LAM but the responses were similar in both the PCR positive and negative groups when challenged with these antigens. Same pattern of response (for both IFN-γ and TNF-α) was observed in PCR positive and PCR negative old MB

and PB cases. But, such comparison needs to be tested with more number of subjects in each subgroups.

When cytokine responses were seen in relation to PCR positivity in healthy contacts of the patients, the IFN-γ responses to 35 kDa antigen and MLSA-LAM were higher in PCR positive contacts than in PCR negative contacts (Table 3). But, the difference was significant only for MLSA-LAM (p<0.05). TNF-α response to 35 kDa and MLCwA was higher compared to response to WML and MLSA-LAM, but, the responses were similar in both the PCR positive and negative healthy contacts when challenged with these antigens.

In subgroups of PCR positive subjects (healthy contacts, old MB patients and old PB patients) we compared cytokine responses (Table 4). IFN-γ and TNF-α response to 35kDa antigen was

Table 2 : IFN-γ and TNF-α response (pg/ml) to four *M. leprae* antigens in PCR positive and negative subjects

Antigen	IFN-γ response (mean±SE)		TNF-α response (mean±SE)	
	PCR +ve subjects (n=30)	PCR –ve subjects (n=15)	PCR +ve subjects (n=33)	PCR –ve subjects (n=17)
35 kDa antigen	1341±164	1333±319	1068±139	986±235
WML	124±43	135±47	122±30	135±36
MLSA-LAM	1150±238	201±78	80±23	56±27
MLCwA	3001±458	1575±549	946±177	791±171

Table 3 : IFN-γ and TNF-α response (pg/ml) to four *M. leprae* antigens in PCR positive and negative healthy contacts

Antigen	IFN-γ response (mean±SE)		TNF-α response (mean±SE)	
	PCR +ve healthy contacts (n=16)	PCR –ve healthy contacts (n=8)	PCR +ve healthy contacts (n=18)	PCR –ve healthy contacts (n=9)
35 kDa antigen	1613±249	1298±216	1413±176	1434±371
WML	53±14	150±49	139±43	191±49
MLSA-LAM	1081±271	376±116	113±39	107±46
MLCwA	2664±506	2770±821	1393±277	1118±240

Table 4 : IFN-γ and TNF-α response (pg/ml) to four *M. leprae* antigens in sub groups of PCR positive subjects

Antigen	IFN-γ response (mean±SE)			TNF-α response (mean±SE)		
	Healthy contacts (n=16)	Old MB patients (n=7)	Old PB patients (n=7)	Healthy contacts (n=18)	Old MB patients (n=7)	Old PB patients (n=8)
35 kDa antigen	1613±249	1186±288	876±235	1413±176	846±342	489±130
WML	53±14	393±144	20±13	139±43	123±82	85±38
MLSA-LAM	1081±271	1126±560	1330±658	113±39	73±38	14±9
MLCwA	2664±506	1683±591	5090±1215	1393±277	569±145	271±102

significantly higher in PCR positive healthy contacts compared to PCR positive old PB patients ($p<0.05$ and $p<0.001$ respectively). Old MB patients showed significantly higher response to WML compared to healthy contacts ($p<0.05$) and old PB patients ($p<0.05$). Old PB patients showed significantly higher response to MLCwA compared to old MB patients ($p<0.05$) and almost reaching significance level compared to healthy contacts ($p=0.05092$). TNF-α response to both 35 kDa and MLCwA antigens was highest in healthy contacts. The response to MLCwA was significantly higher in healthy contacts compared to PCR positive old MB and PB patients ($p<0.01$ and $p< 0.001$ respectively).

Discussion

T cell responses in the subjects residing in the leprosy resettlement village to *M. leprae* specific antigens were estimated using whole blood assays (WBAs). Early detection of *M. leprae* infections would be necessary to plan intervention strategies which in turn would be of great help for leprosy control programs. The WBA is an effective immunological tool to measure *M. leprae* specific cytokine responses in population of endemic area. The detection of *M. leprae* on the nasal mucosa by PCR could indicate possibility of primary nasal infection by *M. leprae*. Systemic immune response in such individuals could further be of interest to know how immune system reacts to such exposures. We observed that the 35 kDa antigen and MLCwA antigen stimulated greater IFN-γ and TNF-α response in all the subjects. These findings were similar to the earlier studies which suggested that the 35 kDa antigen and MLCwA antigen to be major targets of human immune response and elicited good IFN-γ responses (Triccas et al 1996, Weir et al 1998). It was interesting to note that the MLCwA and MLSA-LAM stimulated higher IFN-γ responses in subjects with evidence for the presence of *M. leprae* on the nasal mucosa (PCR positive group). Earlier studies have shown that IFN-γ responses were higher in PB cases and their household contacts (Brennan 2000). Presence of *M. leprae* on the nasal mucosa indicates either transient carriage or sub-clinical infection that may play a vital role in transmission of leprosy (Smith et al 2004). Increased IFN-γ production in subjects with nasal PCR positivity when challenged with *M. leprae* specific antigens could be indication of sub-clinical infection. Earlier studies indicate that cell wall and cytosolic proteins induce Th1 type of immunity which may play a role in protective immunity (Dockrell et al 1996, Manandhar et al 2000). This study showed that MLSA-LAM, MLCwA and 35 kDa antigen induced a good response for IFN-γ whereas 35 kDa antigen and MLCwA showed increased TNF response. Since the number of subjects tested in sub-groups was small, the power to detect statistically significant differences was low. Higher Th1 type cytokine response in subjects with nasal exposure will be important as such response will be important in protection. The WML antigen failed to stimulate significant cytokine response in the subjects but earlier studies have shown that this antigen is very useful in measuring mucosal humoral immune response, in leprosy endemic areas (Smith et al 2004). Our findings suggest that crude *M. leprae* antigen may not be potent cytokine stimulator. This may be due to the disintegration of whole *M. leprae* when exposed to systemic immune response where processed antigens may play role in eliciting specific responses.

The present study indicates that significance of nasal carriage and cytokine responses to different *M. leprae* specific antigens in association with evidence of direct exposure needs to be explored for their possible use in studying infection and early diagnosis of leprosy.

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