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Original Article

Potential of a metabolic gene (*accA3*) of *M.leprae* as a marker for leprosy reactions

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Understanding the mechanism(s) of reactions in leprosy remains a challenging task for both clinicians and basic scientists. While there is some understanding of host processes associated with different type of lepra reactions, there is very little information about bacterial factors triggering these inflammatory processes. This study is continuation of our earlier research programme on leprosy genomics in which significant transcription of 11 genes was observed during active disease and these included *accA3* gene. In present study, we have investigated the potential of this gene or its gene product as molecular and or immunological marker for studying the reactions. Using quantitative Real-Time RT-PCR significant higher expression (mean log2 ratio=3.39) of *accA3* was observed in specimens from leprosy reaction cases compared with cases without reactions. *in silico* homology model of this protein was analyzed for hydrophilic and B-cell epitope regions. Peptides with maximum antigenecity were selected, cloned, expressed and used to study sero-reactivity across the disease spectrum by indirect ELISA. While sero-reactivity was observed in leprosy cases the antibody levels did not vary significantly between the patient/s of same clinical type with and without reaction thereby indicating the limitation of this approach for this purpose. Measurement of transcription of this gene has, thus, potential as a molecular marker for monitoring the reactions.

Key words : Reaction, *acc3*, Marker, Real-Time RT-PCR, *M.leprae*

Introduction

Over the years there have been many advances in our knowledge about the biology of *Mycobacterium leprae* and also management of leprosy. Burden of disease has decreased all over the world by effective use of multidrug therapy (MDT). In India, we have achieved a great success towards the elimination (prevalence rate 0.88/10,000 in July 2006) of leprosy. However, the transmission of the disease is still continuing in some high endemic areas. It is well known that disease process in leprosy is complicated by the episodes of reactions that are acute inflammatory complications often presenting as medical

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emergencies during the course of treated or untreated Hansen's disease. Because *M. leprae* infects peripheral nerves, the inflammation associated with reactions may result in severe nerve injury which may develop rapidly, with subsequent loss of sensation, paralysis and deformity. In earlier period with highly bacillated patients and advanced disease being dominant, these were reported to affect upto 30 to 50% of all leprosy patients (Becx-Bleumink and Berhe 1992, Scollard et al 1994, Kumar et al 2004). Proportion is significantly lower now.

Type 1 known as reversal reaction (RR) occurs mostly in patients belonging borderline portion of the spectrum (i.e. BL, BB and BT) and shows exaggerated delayed type hypersensitivity usually develop gradually and their natural course may last for many weeks. Type 2 reaction, also known as erythema nodosum leprosum (ENL), occurs in multibacillary patients (LL and BL). These reactions have been associated with the presence of high levels of cryoglobulins and immune complexes involving *M. leprae* antigens (Quismorio et al 1978, Drosos et al 1986). While different types of leprosy reactions are known to be immunologically mediated but the mechanisms responsible for different types of reactions remain poorly understood. There is very sketchy information about possible bacterial factors that would be initiating and precipitating this phenomenon. Despite advances in host genomics, no clinical or laboratory test has been developed to accurately predict the possibility of developing reaction in leprosy patients (Scollard et al 2006). In the current scenario, proteins of interest can be prioritized based on potential B-cell or T-cell epitopes; although strict associations between predictions by bioinformatics tools and actual antigenic potential need to be proven by actual studies. While many studies have described novel antigens that show marked humoral and cellular immunogenicity but still their utility in understanding the reactions is limited (Singh et al 1994). Our earlier functional genomic analysis of differentially regulated genes of *M.leprae* in human host led to identification of 11 such genes. In present study, we have analyzed one of these genes; a metabolism associated protein (*accA3*) for its expression levels and sero-reactivity during leprosy reactions. This gene was selected for investigations because of higher expression levels observed in biopsy specimens from reactional lesions.

Materials and Methods

Biopsy specimens from leprosy cases attending the OPD of Medical Unit-I of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (Agra) and Model Rural Health Research Unit, Ghatampur (Kanpur, India) were taken after informed consent and after due approval of Ethical Committee of the Institute. Scalpel biopsy specimens (50-100 mg) were collected in RNALater solution (Ambion Inc., USA) under aseptic conditions. The patients included belonged to tuberculoid type (TT), borderline type (BB), lepromatous leprosy (LL) with and without reactions. Patients were clinically diagnosed and classified according to Indian Association of Leprologists classification (1982) were also grouped as multibacillary (MB=BL/LL) and paucibacillary (PB=BT+BB). Biopsy specimens from healthy (non leprosy) controls undergone for surgery for any reason other than leprosy were obtained from SN Medical College and Hospital, Agra.

Isolation of *M.leprae* total RNA and control RNA

Biopsies were minced with sterile scissors and homogenized properly thoroughly in the pestle mortar to the point the homogenate was able to pass through 26 G needle in Trizol solution (Invitrogen, USA). The smears were prepared from homogenate, stained with Ziehl-Neelsen (ZN) stain and examined for acid fast bacilli (AFB) which were also counted as per method of Shepard and McRae (1968). Total RNA including *M.leprae* RNA was extracted from skin biopsy homogenates using manufacturer's protocol. DNA was removed with DNase I using DNA-

free™Kit (Ambion Inc., USA) as specified by the manufacturer.

Relative expression of *accA3* in leprosy samples with and without reaction

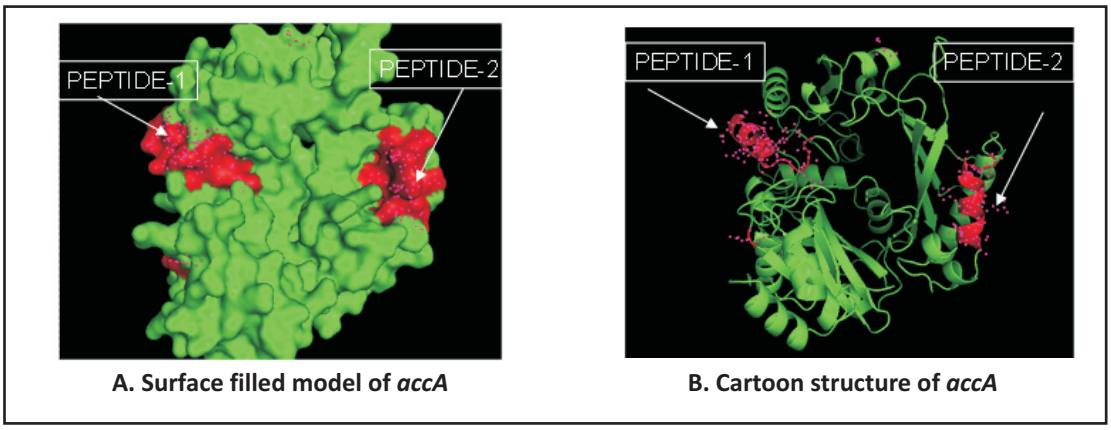
Total live bacterial load in each sample was calculated by using QRT-PCR targeting 16S rRNA (Sharma et al 2008) and RNA was diluted equivalent to 1.0E+2.0 bacilli / ml of homogenate so that expression could be compared from same number of bacilli. Relative expression of *accA3* genes was compared within same clinical type having reaction by Real-Time RT-PCR using Light Cycler RNA Amplification Kit SYBR Green 1 (Roche Diagnostics, Germany) with 200 nM of each gene specific primer. Relative expression of *accA3* gene was analyzed in cases of same clinical type with reaction and without reaction. Reverse transcription at 55°C for 10 min and initial denaturation at 95°C for 30 sec was followed by 45 amplification cycles of annealing at 59°C and extension at 72°C with ramping of 20°C/Sec. Single step Real-Time RT-PCR using SYBR Green chemistry was performed including at least one positive control of known copy number of template and RNA extracted from healthy (non leprosy) were used as negative control, data were normalized with 16rRNA gene and converted in to linear fold change and log2 ratio.

Bioinformatic analysis

As crystal structure of this gene is not available for further study, homology based energy minimized (-4729.011 KJ/mol for *accA3*) 3D model (Figure 1) of this protein was generated by using structures having more than 40% identity through Swiss PDB server (http://www.pdb.org.in). Protein sequence was screened with Peptide Antigen Finder Tool (http://www.proteinlounge.com/pepfinder_home.asp) to find out the best antigenic peptides of a protein sequence (maximum hydrophilic regions of protein sequence) which would most likely be found on the outer surface of protein. Epitope mapping of PDB structure to predict B-cell epitopes was performed using any of the physico-chemical properties (hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns). The peak of the amino acid residue segment above the threshold value (default is 2.38) were considered as predicted B-cell epitope (Table 1). Two best scoring peptides (92-LAENADFAQAVIDA-106, 406-LARARRALDEFEVE-419) were got commercially synthesized from MultiSynTech© GmbH, Germany. Two coding sequences were cloned and expressed.

Cloning and expression of identified peptides

Two regions coding for above selected peptides



A. Surface filled model of *accA* **B. Cartoon structure of *accA***

Figure 1 : Homology structure of *accA* protein showing epitopes hydrophilic (peptide 2) and B-Cell epitope (peptide 1) used for study sero-reactivitty in leprosy patients.

Table 1 : Physico-chemical properties of peptides selected for cloning and sero-reactivity

Peptide	Length	Region	Molecular weight, MW
PEP-1	130	180 to 310	14732.5
PEP-2	100	450-550	10611.9

were amplified, cloned and expressed in *E. coli* by using Zero Blunt Cloning Kit (Invitrogen Inc., USA) as per manufacture's protocol. Transformed *E. coli* cells containing the insert (selected by amplification with direction specific primers) as well as same strain of *E.coli* were lysed by ultrasonication. Total protein content of transformed as well as non transformed *E. coli* cell lysate was estimated by Bradford method. Equal amount of protein lysates (40 µg total protein) from each clone and non transformed *E.coli* cell were electrophoresed on 12% SDS-Polyacrylamide gel. Clear bands of cloned peptides with estimated molecular weight were observed (PEP-1 of 14.7 kD and PEP-2, 10.6 kD) in transformed *E. coli* cell lysates whereas no band of same size was present in non transformed *E. coli* cell lysates (Figure 2). Thus, both the peptide coding regions were successfully cloned and significant expression in *E.coli* was observed.

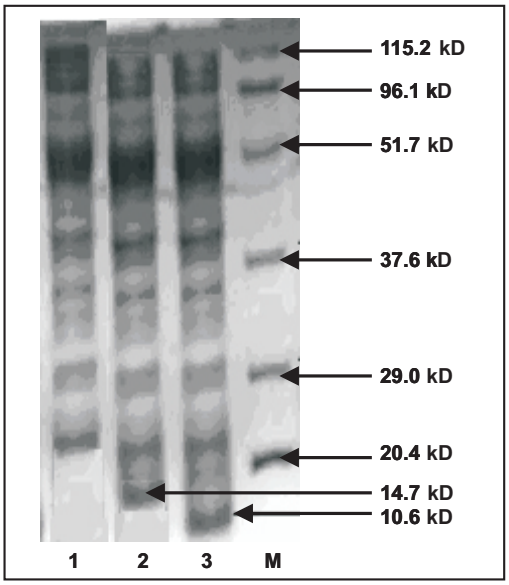


Figure 2 : SDS-PAGE electrophoresis of cell lysate transformed and transformed non transformed *E.coli* bacterial cell.

Lane 1 : Non-transformed *E.coli* cell Lysate, **Lane 2 :** Transformed *E.coli* cell lysate showing over expression of antigenic region (PEP-1= 14.7 kD), **Lane 3 :** Transformed *E.coli* cell lysate showing over expression of antigenic region (PEP-2=10.6 kD), **Lane 4 :** Molecular weight marker

Sero-reactivity of *accA3* (LM0726)

A total of 40 (10 BL/LL + 10BB/BT + 10 reaction (5RR+5ENL) + 10 healthy) were studied. Blood samples (5 ml) each were collected by from different types of leprosy patients including reactions from patients attending the OPD Unit-I of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (Agra) and Model Rural Health Research Unit, Ghatampur (Kanpur, India) serum was separated. Indirect **e**nzyme linked immune sorbent assay (ELISA) was performed for detection of antibodies against synthetic peptide and recombinant protein of ML0726 gene of *M. leprae*. The microwells of ELISA plates were coated with (50 µl/well of 5 µg/ml) each of synthetic peptide as well as recombinant protein and kept at 40°C for overnight. *M. leprae* cell lysate was also coated in one lane of ELISA plate as positive control and non recombinant *E.coli* cell lysate was taken as negative control in every experiment after blocking (1 % BSA) and washing (with PBST) serum dilutions of 1:50 and 1:100 were added (50 µl/well) and incubated at 37°C for 2 hrs. After washing 50 µl of peroxidase labeled anti- human IgG (diluted in TBS-T in 1:6000 ratio) was added in each well and incubated at 37°C for 1 hr. Plate was washed, substrate was added followed by stopping of reaction with 10%, H_2SO_4 and absorbance was measured at 450 nm in ELISA reader. Absorbance at 450 nm while using *M. leprae* whole cell lysate as antigen was considered as positive control and non transformed *E.coli* cells were used as negative control antigen.

Results

Quantitative expression profiling of *accA3* (ML0726) gene across the disease spectrum

In Real-Time RT-PCR experiments *accA3* was observed to be significantly over expressed in specimens from reaction cases compared with cases of similar clinical type. Hyper expression of *accA3* was observed in both types (mean log 2 ration=3.39) of cases during the reaction as compared same clinical type without reaction (Table 2). Analysis of equality of variants (1. BT/BB; 2. BT/BB with reversal reaction; 3. BL/LL and 4. BL/LL with ENL reactions) was performed and over all significant difference (P=0.002) was observed by using Levene's test and ANOVA analysis by using SPSS statistical software. Post Hoc tests (one way ANOVA) was applied for pair wise comparisons of variants (clinical groups) of *accA3*. Relative expression of *accA3* (P=0.003) was found to have significant difference (P<0.05) between variant 1 (BT/BB) v/s 2 (BT/BB with reversal reaction). Similarly in cases having ENL i.e. variant 3 (BL/LL) v/s 4 (BL/LL with ENL reaction there was significant (P=0.006) difference in expression of this gene. We observed > 10 fold over expression during the reaction compared to bacilli from same clinical type patient having reaction by quantitative RT-PCR.

Study of sero-reactivity of *accA3* (ML0726) by using ELISA

Synthetic peptides as well as recombinant protein of *accA3* gene showed reactivity with sera from patient across the disease spectrum. Stronger sero-reactivity with sera of BL/LL leprosy patients was observed (mean of OD at 450 nm =1.544±0.109) compared to BT/BB leprosy patients (mean of OD at 450 nm =1.23±0.07). No significant difference (P>0.05) in sero-reactivity was observed between BT/BB cases with reaction (OD at 450 nm =1.23±0.06) and without reaction (OD at 450 nm =1.35±0.04) of same clinical type (BT/BB) of leprosy. Similarly in BL/LL cases there was no significant (P=0.17) difference in sero-reactivity was observed between cases with reaction (OD at 450 nm=1.19± 0.02) and without reaction (OD at 450 nm =1.23±0.07).

As represented in Figure 3 repeated measures ANOVA with Bonferroni's multiple comparison analysis was performed to compute significance of reactivity of each peptide in four 3 clinical types (4 variants including healthy control). Significant difference (P<0.05) between reactivity of this protein with sera from healthy (mean OD at 450<0.30) individuals and BT/BB (absorbance=1.23±0.07) as well as BL/LL (absorbance=1.45-1.68) was observed. There was no statistically significant difference in the reactivity of non recombinant *E. coli* cell lysate (P>0.05) with sera from healthy v/s any type of leprosy patients. *M. leprae* whole cell lysate showed significant difference (P<0.001) in reactivity against sera from healthy individuals v/s leprosy patients. However, there was no significant difference (P>0.05) between leprosy patients with reaction (absorbance = 1.18-1.30) and without reaction (absorbance =1.25-1.60) against any peptide of this protein in BT/BB as well as BL/LL cases (Figure 3).

Table 2 : Relative quantification of *accA3* gene expression by 2^{-Ct} method (Livak and Schmittgen 2001) in different clinical type of leprosy patients

Disease type	Mean ± SD crossing over point (Ct value)		Ct	Fold Change	Log2 fold change
	No reaction	Reaction			
BT/BB/ Borderline leprosy	21.642± 0.54	18.174±0.387	3.468	11.06553	3.468
BL/ LL Lepromatous leprosy	21.51±0.87	18.19±0.39	3.321	9.986644	3.32

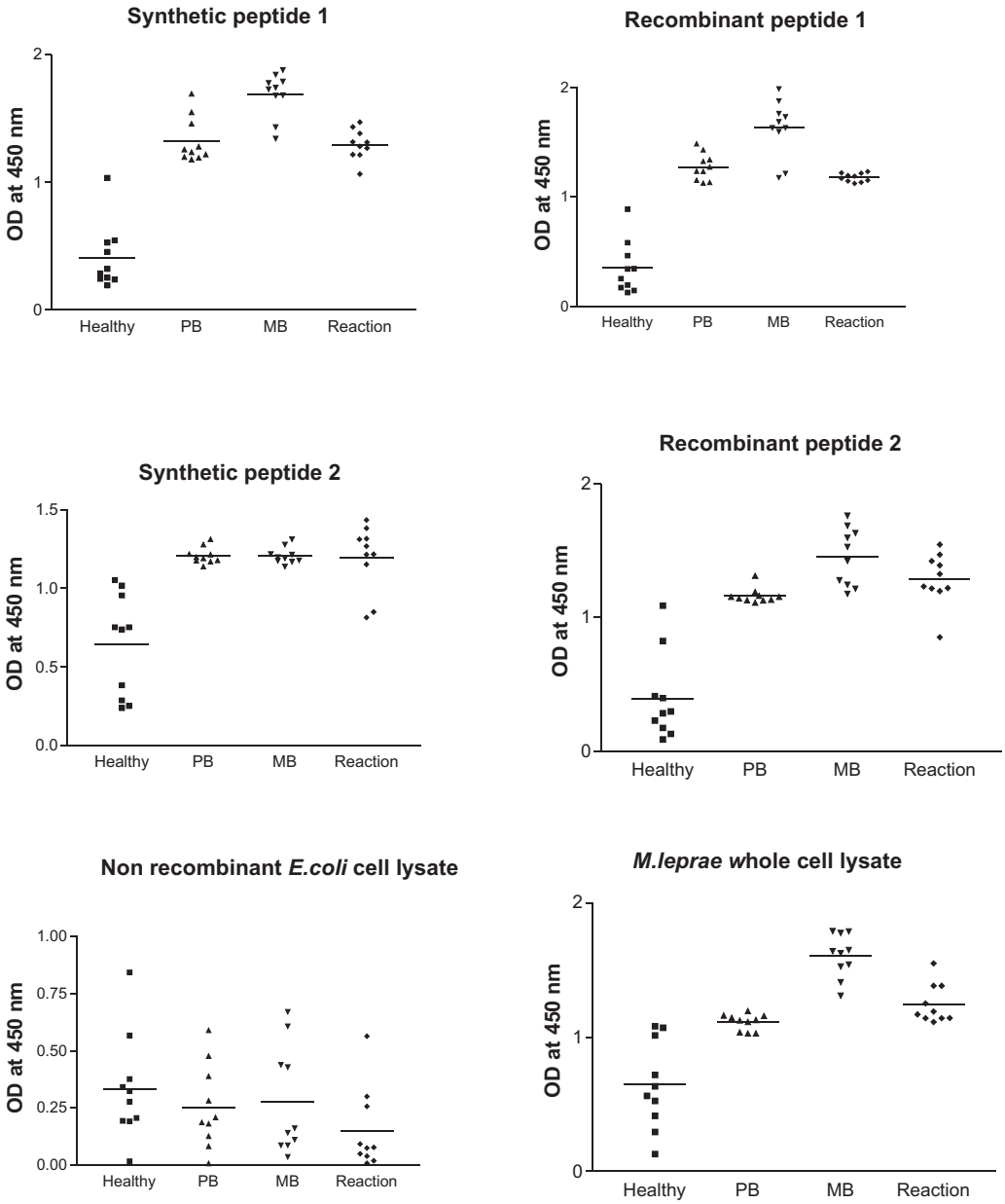


Figure 3 : Sero-reactivity of *accA3* gene in different types of leprosy cases (PB= BT/BB; MB= BL/LL) against selected antigenic regions.

Discussion

Different types of reactions in leprosy appear to have different underlying immunologic mechanisms but there is considerable overlap and the divide between ENL as reversal reactions is not that clear cut as was originally believed. As the bacterial factors that initiate them are largely unknown, intensive search has continued over the years to understand the role of different host and bacterial factors in the pathogenesis of reactions in leprosy.

In the recent years, bioinformatics and experimental approaches have been used to evaluate various *M. leprae* proteins or small sets of proteins as potential sero-diagnostic or T-cell antigens (Geluk al 2005, Aráoz et al 2006). By such approaches a number of new proteins Ml0405 and Ml2331 showing hyper reactivity have been identified (Reece et al 2006), some have been used to enhance serological detection with PGL-I (Geluk et al 2005). In a study, different recombinant proteins and 58 peptides (9 mers and 15 mers) were tested for IFN- responses in peripheral blood mononuclear cells (PBMC) from leprosy patients seeking epitopes that would increase specificity and observed shorter proteins (15 mers and 9 mers) are more specific but less sensitive than four recombinant proteins (Ml0008, Ml0126, Ml1057 and Ml2567) for IFN- production (Spencer et al 2005). Early functional studies of lymphocytes demonstrated increased lymphocyte proliferation in response to *M. leprae* antigens *in vitro* during type 1 reactions (Godal et al 1973, Barnetson et al 1976). However, the methods for selecting and screening of potential antigens in these studies were dramatically different. These studies did not lead to identification of any antigens that could distinguish reactional cases from others in the similar spectrum.

In our earlier study using an indigenously developed DNA Chip (Indian Patent Application No. 2012/DEL/2006 and No. 884/DEL/2007), Acetyl CoA carboxylase (*accA3*) synthase was found to be >10 fold over expressed (transcribed) during the reactions in borderline as well as lepromatous leprosy. It was hypothesized that this may be a defensive reaction of pathogen. It was thought that reactivity of host immune system to this protein or its epitopes may have role in the immunopathology as well. Peptide sequences of *accA3* having antigenic property were used to study the sero-reactivity of different types of leprosy patients. There was sero-reactivity to these peptides as well recombinant proteins in BT/BB, BL/LL cases but there no difference between sero-reactivity of cases having reversal reactions/ENL versus cases without reactions. This protein thus merits to be investigated in a statistically significant number of specimens from leprosy as other mycobacterial diseases to confirm its diagnostic potential. However, its usefulness as a prognostic marker does not appear to be good. As selected epitopes of this protein have shown sero-reactivity with leprosy patients so this antigen / epitope may be evaluated to monitor the progress of disease after confirmation of reactivity on adequate number of patients across the diseases spectrum. On the other hand, assessment of transcription levels of this gene indicates its usefulness as a potential molecular marker for monitoring reactions in leprosy. Considering its consistent over expression across the disease spectrum this gene appears to be a good potential target for studying viability of *M. leprae.*

Hydrophilic, B-cell epitope surface peptide sequence and recombinant proteins generated have been used the *in vitro* study of mechanism of reaction in the leprosy by using indirect ELISA to detect the differential antibody titer in the different clinical types and reactivity with antibody in host were observed even in PB (BT/BB) patients also. Over expression during the reactions in the leprosy patients as compared to other disease types suggests the activation of this part of metabolism during leprosy reaction. Presence of antibodies against this cytosolic protein suggests that such antigens may be contributing to delayed type of hypersensitivity (DTH) during reversal reactions (Cooper et al

1989). Low reactivity observed in healthy individuals may be due to shared homology of this selected peptide with some common organisms present in the body or background due to exposure to *M.leprae* or related mycobacteria. On the whole this protein does not appear to be a good prognostic marker.

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