MUTATION IN ALKYLHYDROPEROXIDASE D GENE DRAMATICALLY DECREASES PERSISTENCE OF *MYCOBACTERIUM BOVIS* BACILLUS CALMETTE-GUERIN IN INFECTED MACROPHAGE

TAGHI NASERPOUR FARIVAR[1], POURAN JOHARI VARNOUSFADERANI, ABASALT BORJI[1]

ABSTRACT

BACKGROUND AND OBJECTIVES: Mycobacterium tuberculosis is the leading cause of death from a single bacterial species in the world and is subjected to a highly oxidative environment in its host macrophage and consequently has evolved protective mechanisms against reactive oxygen and nitrogen intermediates. Alkyl hydroperoxidase D (AhpD) is a molecule from these mycobacterial defense systems that has a dual function. It not only works with Alkyl hydroperoxidase C (AhpC) in mycobacterial defense system against oxidative stress but also has a role in oxidation/reduction of succinyltransferase B (SucB), dihydrolipoamide dehydrogenase (LPD) and AhpC. The present study was undertaken to find out the effects of inactivation of ahpD gene in the intra-macrophage persistence of resulted BCG mutant. MATERIALS AND METHODS: We did allelic exchange mutagenesis in Mycobacterium bovis BCG and evaluate the effects of this mutagenesis in intracellular persistence of wild type BCG strains and ahpD mutant ones by comparing colony forming units (CFU) in infected macrophage. RESULTS: Our findings showed that after producing allelic exchange mutagenesis in ahpD gene of M.bovis BCG a sever decrease in the CFU's of ahpD mutant BCG strains has been observed and intracellular persistence of ahpD mutant BCG strains decreased significantly. CONCLUSION: Mutagenesis in ahpD gene will cause significant decrease in intracellular survival of ahpD mutant strains than wild type M.bovis BCG strains and could leads to an inefficiency in pyruvate dehydrogenase pathway and could also impair impairs mycobacterial defense system against oxidative and nitrosative stress.

Key words: AhpD, macrophage, mycobacteria, Mycobacterium bovis, persistence

[1]School of Medicine, Research Institute for Tropical Medicine and Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran

Correspondence:
Dr. Taghi Naserpour Farivar
School of Medicine,
Research Institute for Tropical Medicine and Infectious Diseases, Zahedan University of Medical Sciences,
P.O. Box 98165-506,
Zahedan, Iran.
E-mail: taghin@yahoo.com

INTRODUCTION

In most infected individuals, oxidative and nitrosative stress controls proliferation of *Mycobacterium tuberculosis* in infected macrophage[1,2] and in these host macrophages, *M. tuberculosis* is subjected to a highly oxidative environment.[3] In this situation, produced

peroxynitrite (ONOO-) and other reactive nitrogen and oxygen intermediates plays an important role in host defense against the invading bacteria.[4,5] Consequently, many bacterial pathogens have evolved protective mechanisms against reactive oxygen and nitrogen intermediates.[6]

On the other hand, it has been cleared that mutation in the catalase-peroxidase *katG* gene of *M. tuberculosis* resulted in resistance to isoniazid[7] and mutation in the flavoprotein mono-oxygenase *etaA* gene, resulted in resistance to ethionamide.[8]

Interestingly, although KatG and EtaA have different activating enzymes, mutations of *katG* and *etaA*, resulted in elevated expression of alkyl hydroperoxidase C (AhpC)[7] and analysis of the genes induced in isoniazid-resistant *M. tuberculosis* strains indicated that up-regulation of *ahpC* gene, is one of the mechanisms used by the organism to restore the loss of the KatG protein antioxidant activity.[9]

AhpC is a member of the ubiquitous peroxiredoxin family and its disulfide bond is reduced by different mechanisms to give a sulfenic acid (-SOH) intermediate in different organisms.[10] In *M. tuberculosis* thioredoxin and thioredoxin reductase, do not reduce the corresponding AhpC. Previous studies showed that an alkyl hydroperoxidase D (*ahpD*) gene (Rv2429) coding for AhpD protein with no sequence uniqueness to AhpC is located instantly next to the *ahpC* gene and it works as reducing partner of AhpC. AhpD is reduced by dihydrolipoamide succinyltransferase (SucB) and dihydrolipoamide dehydrogenase (LPD).[11] AhpD is a homo trimer and contains two cysteines which are required for enzymatic activity of AhpD protein.[12]

Thus, AhpC, AhpD, SucB, and Lpd together constitute a peroxidase active toward both hydrogen and alkyl peroxides.[13] These studies have yielded a potent *in vitro* inhibitor of AhpD that has been used to explore the potential of AhpD as a target for antituberculosis drug development.[14]

These findings showed that AhpD has a dual role in metabolic pathway of pyruvate dehydrogenase and in anti-oxidative pathway of mycobacteria. As low titer of AhpD suffices to maintain AhpC activity and known inhibitors of AhpD do not completely suppress the *in vitro* activity of AhpC/AhpD, using competitive precursor for AhpD could not inactivate the AhpC/AhpD complex.[14] So, we did this study to determine the effects of allelic exchange mutagenesis in *ahpD* gene on the persistence of *Mycobacterium bovis* Bacillus Calmette-Guerin(BCG) in infected Macrophages.

MATERIALS AND METHODS

Materials

All chemical reagents were purchased from Merck and Sigma (Tehran, IRAN). *Escherichia coli s*train BL21 (DE3) was from Fermentas, (Vilnius, Lithuania). Oligonucleotide synthesis was done by DNA Technology Co, (Copenhagen, Denmark) and Bio-Rad Mini MJ DNA thermal cycler was used for PCR experiments (Bio-Rad, Tehran, Iran).

Media and growth conditions

The strains were grown until mid-exponential phase and/or stationary phase (as indicated)

on 7H9 (Difco) or 7H11 Middlebrook media, supplemented with 0.5% Tween, 0.2% glycerol and10%OADC (oleic acid, bovine serum fraction V, glucose and catalase). Bacteria were grown at 37 °C. All manipulations of live *M. bovis* BCG Pasteur strain 1173P2 were carried out under Biosafety Level III conditions.

Cloning and allelic exchange mutagenesis

PCR amplification of the *ahpD* gene was performed using these primers; forward: 5'-GATCTGGTTGCCCGG AACATATGAGTATAGAAAAGCTC-3'; reverse: 5'-GGCGTCATGGCGTCGACACACTTAGCTT GGGCTTAGTGCCTCGGTTGTGCC-3

The reaction contained 50 ng of *M. bovis* BCG Pasteur strain 1173P2 genomic DNA, 50 pmol each of the primers, 2 mM dNTPs, and 10 units of Vent DNA polymerase in a final volume of 100 µl of 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 2% dimethyl sulfoxide, and 0.1% Triton X-100. The annealing and extension cycles were as follows: 90 °C for 10 min (1 cycle), 90 °C for 1 min, 72 °C for 1 min, 60 °C for 1 min (30 cycles), and 72 °C for 10 min (1 cycle).[15]

Disruption of *ahpD* gene: we disrupted *ahpD* gene in *M. bovis* BCG Pasteur strain 1173P2 by using the gene replacement and transposon delivery strategy by Guilhot[16] method. Briefly, following gel purification of the amplified *ahpD* gene, 0.55-kilobase product gene was digested with PstI and the ligated two resulted fragments with hygromycine resistance cassette resulted from digestion of pGoal 19 with pstI. Then we cloned the ligated fragment into pPR23(*ts-sac B* vector) with cutting this plasmid by KspAI (pPR23ahpD plasmid). Resulted plasmid electroporated into *M. bovis* BCG Pasteur strain 1173P2 by gene pulser(Bio-Rad,Tehran,Iran) with the following conditions; Single Pulse; 2.5 KV;1000 Ohms;25µF[16] and then we selected mutant BCG by culturing them on hygromycin containing media which had 2% sucrose. After culturing mutant strains in medium A for 14 days at 37°C, 2 ml of liquid medium was selected and after centrifugation at 8000 rpm for 10 min, total RNA of isolated bacteria was purified by Max kit (Ambion, Austin, USA) and underwent for Real Time PCR.

Real time PCR

The selected ahpD gene primer and probe sequences were as follows: forward primer F3, 5'- TGGCGGGAATCAATGAG-3', and reverse primer 5'- GCTTGATGTCCTTGGCGTACTC-3'; and probe 5'-R- AAAGCTCAAGGCCGCGCTCCC -Q-3' designed with Primer Express software (Applied Biosystems).The probe is labeled at its 5'end with the reporter dye (R) FAM (6-carboxyfluorescein) and at the 3'end with the quencher dye (Q) TAMRA (6-carboxytetramethylrhodamine). The primers and probe were prepared by DNA Technology A/S (Copenhagen, Denmark).

Real-time PCR was performed using an ABI Prism 7500 System (Applied Biosystems, Foster City, CA).Each 50-µl reaction mixture consisted of 40 µl of PCR mix combined with 10 µl of internal standard, control, or specimen DNA. The PCR mix contained universal master mix (Applied Biosystems), primers forward and reverse (each at 50 nmol/reaction), probe (10 nmol/reaction), and 13 µl of RNase/DNase-free water. After 2 min at 50°C and 10 min at 95°C, there were 45 cycles (95°C for 15 s and 60°C for 1 min) of PCR amplification for AhpD gene

detection. AmpErase and dUTP within the master mix provided carryover contamination control.

Bacterial cell association and replication

Suspensions of bacteria were prepared in 7H9 broth, and the optical density at 600 nm was adjusted to 0.5 (~10^8 CFU/ml).[17] Prior to macrophage infection, all mycobacterial preparations were pelleted, washed, declumped by two passages through an 18-guage needle and three passages through a 22-gauge needle, and centrifuged at 150 x g for 5 min to remove clumps.[18] 10 µl of this suspension was added to each well, and the plates were incubated at 37°C in 5% CO_2 .The viability of the organisms was determined by plating serial dilutions of the infecting inoculums on 7H10 agar.[17] Viability ranged from 70 to 84% in these experiments.

Human peripheral blood mononuclear cells

Heparinized blood from healthy blood donors was diluted 1:1 with 0.9% saline, and the mononuclear-cell fraction was obtained by centrifugation at 800 x g for 30 min at 24°C over a Ficoll-sodium diatrizoate solution (Ficoll-Paque; Pharmacia Fine Chemicals). The layer containing the mononuclear-cell fraction was removed and diluted 1:1 with RPMI 1640, and the mononuclear cells were collected by centrifugation at 400 x g for 10 min at 4°C. The mononuclear cells were washed twice by centrifugation at 115 x g for 10 min at 4°C. The cells were resuspended in RPMI 1640, counted in a hemocytometer, and adjusted to a concentration of 1.5 x 10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated (HI) FBS and 10% autologous serum, and 1.0 ml was added to plastic (Bavaria Medico, Germany) cover slips in 2 cm^2 tissue culture wells (Falcon, Becton Dickinson, Lincoln Park, N.J).[19] The participation of normal human blood donors in our research was approved by Zahedan University of Medical Ethics Review Board.

Cell suspensions of the *M. bovis* BCG Pasteur 1173P2 and mutant BCG were added to the attached macrophages at a multiplicity of infection (MOI) of 1:10 (1 bacterium per 10 host cells). Each day, the infected macrophages were washed twice with Hank's Balannce Salt Solution (HBSS) and overlaid with fresh Iscove's Modified Dulbecco's Medium (IMDM).[17]

CFU assay: Adherent monolayers were disrupted with a solution of water containing 0.016% Digitonin and 0.25% Tween 80 (Sigma Chemical Co.). Bacterial suspensions were serially diluted and plated onto Middlebrook 7H10 agar plates supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco). Plates were incubated for three hours, one day, eight days and 15 days at 37°C. Colonies were counted under a dissecting microscope and reported as CFU. For each culture dilution, six replicate samples were plated and the mean number of colonies was calculated.[20]

Stimulation of Growth of *ahpD* mutant: Stimulation of growth of *ahpD* mutant was done by adding the following substances: Thiamine 150 µg/ml, Thymine 8 µg/ml, Guanine 45 µg/ml, Adenine 920 µg/ml, Sodium acetate 3.8 mg/ml, Succinic acid 5 mg/ml.[21]

Almar Blue

Macrophage plated in 12 well plate in the range of 2x10^5 cells per well and infected with 10 fold BCG for one hour in 37ºC (control and mutant).

These infected cells then washed 2 times with PBS and lysed by adding 1 ml of cold water. Lysates were transferred to a clear 96 well micro plate which was sealed by parafilm and 15 micro liters of 10% Almar Blue reagent (BioSource Intl.) and 10% Tween 80 were added to 200 micro liters of lysed cells. Then micro plate incubated at 37°C and the emission was read at 590 nm.

RESULTS

Mutagenesis in *ahpD* gene was confirmed by Real Time PCR of the Wild and *ahpD* mutant strain after total RNA extraction from mutant and wild type BCG culture and reverse transcription [Figure 1]. This mutagenesis resulted to production of *ahpD* mutant BCG strains which were not able to grow in routine mycobacterial culture media (Middlebrook 7H9 or 7H10) as well as do wild type strains. These mutants were only able to growth in these media in the presence of supplementary materials. So, like previous experiments,[21] we added supplements to culture media to enhance mutant growth rate.

We added supplements into our three BCG mutant test groups and maintained one group without adding supplements as control. The maximum growth was observed in group A medium which contained all kinds of supplements mentioned in Table 1, followed by the group B, which had not contain succinic acid and sodium acetate and the least growth was observed in group C which had only succinic acid and sodium acetate [Table 1]. Growth curve of wild and *ahpD* mutant BCG strains were showed in Figure 2.

Intracellular persistence of both wild and mutant BCG strains were analyzed by counting viable CFU after one, eight and 15 days of infection in six different wells for each group and the results are mean of these amounts. As described in Figure 3, both strains failed to grow, but they were not killed at similar rates by the macrophages. These results for kinetics of wild type intracellular BCG agree with those from previous studies.[22] Direct observation of the cells with acid fast staining (Kinyoun) demonstrated that the percentage of cells associated with mycobacteria (37%) were the same for wild type and mutants but the number of the two strains during the incubations was different. Taken together, these results show

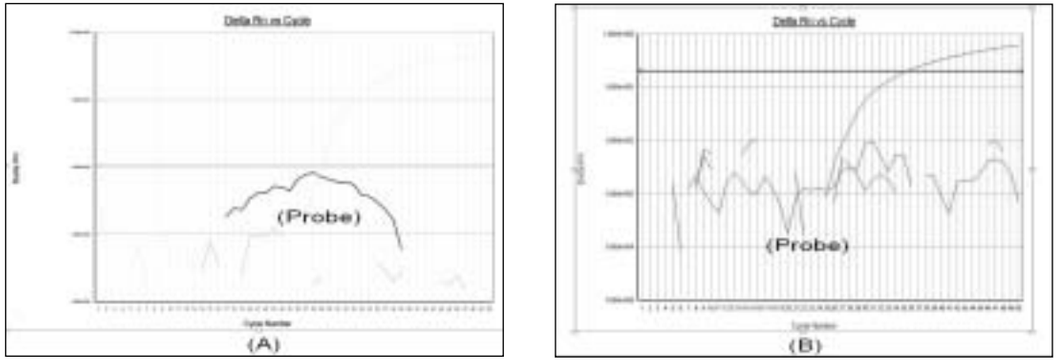

Figure 1: Real Time PCR amplification plots of ahpD gene in ahpD mutant (A) and Wild type BCG (B) strains

Table 1: Supplemented materials to enhance growth of ahpD Mutant BCG Strains

Components / Medium							
Medium A	+	+	+	+	+	+	+
Medium B	+	–	–	+	+	+	+
Medium C	+	+	+	–	–	–	–
Medium D	+	–	–	–	–	–	–

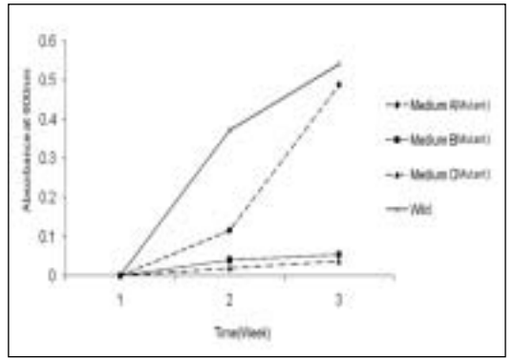

Figure 2: Growth Curve of Wild and Mutant BCG (Pasteur Institute) in Different Culture Media

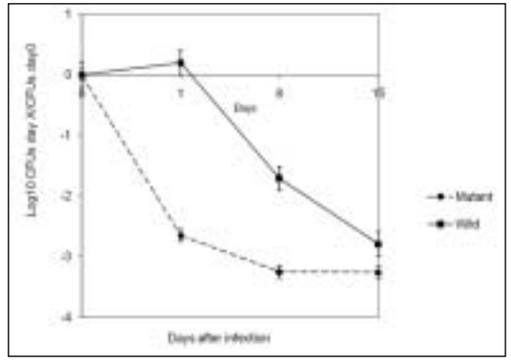

Figure 3: Survival of wild (Pasteur Institute) and ahpD mutant BCG strains in cultured macrophages. After 3 hours of infection, BCG CFU counts were determined from infected cell lysates

that mutagenesis in *ahpD* gene significantly affects BCG growth inside infected cultured macrophages.

Our study showed that after a transient increase in the CFU of lysates of the infected macrophage by the wild type BCG strains in the first day, the CFU of these lysates decreased in the next days. On the other hand, *ahpD* mutant strains in addition to the growth problems which mentioned before, were killed rapidly in infected macrophages in such a way that their CFU's immediately decreased from the first day and in the fifteenth day reached to its least count number. The ratio of CFU's of *ahpD* mutant strains to wild type one's decreased 700 and 45 fold in days one and eight respectively and 60% fold in day fifteen [Figure 3] and so, there is a significant relation between mutation in *ahpD* gene and decreasing survival of the resulted mutants. Also Almar Fluorescence test showed 0.03, 2.5 and 60 percent mean decrease in the fluorescent emission of wild than *ahpD* mutant BCG in first, eight and fifteen days respectively [Figure 4].

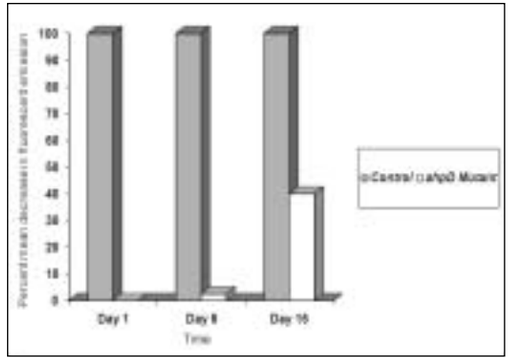

Figure 4: Human Peripheral Blood Monocyte-Derived Macrophage-BCG-Almar Blue

DISCUSSION

Our study showed that mutation in *ahpD* gene make mutant strains susceptible to intra-cellular killing power of infected macrophage. Also, our study showed that mutation in *ahpD* gene make mutant strains unable to growth in routine mycobacterial culture.

We did this study to find whether mutation in *ahpD* decrease intra-cellular survival of mutant strains. For evaluating this question, we did allelic exchange mutagenesis in *ahpD* gene of *M.bovis* BCG and then we infected macrophage culture with wild and mutant BCG strains and evaluated the intra-cellular survival of these strains by colony counting and Almar blue assay in six wells for each group.

We compared means of colony count CFU and Almar fluorescent of wells in wild and mutant BCG strains. Analysis of these data showed that there is a significant relation between mutation of *ahpD* gene and decrease of intra-cellular survival of wild and mutant BCG strains.

Our study showed that *ahpD* not only take part in the oxidative and nitrosative resistance mechanisms of BCG but also, it has a critical role in the growth of BCG strains in routine mycobacterial culture. Also, our findings showed that there is some substitutive mechanism(s) that make mutant BCG strains to survive in infected macrophage although very weak.

On the other hand, Koshkin study[14] showed that *ahpD* is an element of peroxiredoxin defense against oxidative stress. Also Hillas and his colleague[15] reported that the *ahpC* and *ahpD* are anti-oxidant defense system of *M. tuberculosis*.

As Bryk and his colleague[13] reported, if we could be able to inhibit SucB or Lpd in *M. tuberculosis* without affecting their human counterparts, both the Krebs cycle and the bacillus's ability to synthesize acetyl-coenzyme A (CoA) from endogenous precursors could be in danger and our study suggests that AhpD is a good candidate for this purpose.

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