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Expression of Alpha-Crystalline Protein in the In- Vitro Model of Hypoxically Induced Nonreplicating Persistence of Mycobacterium Tuberculosis Strains

Mohammad Najafi-Mosleh ¹, Parisa Farnia ², Kumars Ghazi Saidi ¹, Foroozan Mohammadi ³, Mohammad Reza Masjedi ⁴, and Ali Akbar Velayati ⁵

¹ Department of Medical Bacteriology, Division of Pathobiology, Faculty of Health, Institute of Health Research, Tehran University of Medical Sciences and Health Services ² Department of Mycobacteriology, ³ Department of Clinical Anatomical Pathology, ⁴ Department of Pulmonary Medicine, ⁵ Department of Pediatrics, NRITLD, Shaheed Beheshti University of Medical Sciences and Health Services, TEHRAN-IRAN.

ABSTRACT

Background: Latent tuberculosis infection (LTBI) is caused by *Mycobacterium tuberculosis* (MTB) in a state of non-replicating persistence (NRP). Recent evidence suggests that some very specific adaptations to oxygen depletion occur so that MTB undergoes hypoxic NRP state. In this study the modified slowly stirred, limited Head Space Ratio (0.5HSR) method was used to investigate the physiological response of MTB to different oxygen tension levels.

Materials and Methods: For setting up the various NRP stages, some susceptible and drug resistant clinically isolated strains of MTB were cultivated in Dubos Tween-Albumin medium via hypoxically, slow stirring 0.5 HSR methods. Additionally, the effects of isoniazid, rifampin, pyrazinamide, ciprofloxacin and metronidazole against MTB were examined during NRP-1 and NRP-2 stages. The α -crystalline protein was detected during NRP-1 stage of the MTB cultures via performance of the suitable procedures for pellet preparation, washing and cell disruption and SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) technique.

Results: NRP-1 and NRP-2 stages of MTB were assessed. The first three of the four drugs mentioned above affected the MTB at actively replicating period and the rifampin effect was continued slightly during NRP-1 stage. Metronidazole affected the MTB at anaerobic NRP-2 stage. Alpha-crystalline protein was detected in NRP -1 stage but was not detected at aerated cultures.

Conclusion: Induction of the α -crystalline protein during hypoxic shift-down of MTB metabolism, and its function as a chaperone, suggests a critical role for this protein in the ability of MTB to persist without replicating in the hostile regions of the host's tissues. Therefore, for an effective TB control program, it is critical to understand the mechanisms of factors induction associated with the hypoxic condition of tubercle bacilli and some strategies for the identification of new drug targets must be developed and the persistence states in human lesions should be prevented as well. (*Tanaffos* 2005; 4(14): 43-52)

Key words: Alpha-crystalline protein, Hypoxically, Mycobacterium Tuberculosis, NRP-1, NRP-2

Correspondence to: Najafi Mosleh M

Address: Department of Medical Bacteriology, Tehran University of Medical Sciences, Fax: +98-21-22291203

Email address: n-mosleh@yahoo.com

INTRODUCTION

Latent tuberculosis infection (LTBI) is defined as a clinical condition without clinical or radiological signs of active disease. Approximately two billion people, or one-third of the world's population, have LTBI, and it is estimated that about 10% of them will develop active TB during their lifetime. It is also estimated that one-third of all new cases of active TB result from activation of LTBI. The role of LTBI is even greater in persons co-infected with HIV. Currently, 11 million people in this category are registered throughout the world, and at least 10% of them develop active TB every year (1). Data have shown that the basis for LTBI in humans is persistence of tubercle bacilli for long periods of time even throughout the life. This status is currently defined as dormancy or non-replicating persistence (NRP). Besides LTBI, a part of bacterial population in patients with active disease also persists in a non-replicating state, and there is an obvious need for effective drugs for eradication of this subpopulation. There is evidence of both macrophage physiology and the nature of TB granuloma which suggests that hypoxia is a major factor for inducing NRP state of tubercle bacilli. There is evidence that some very specific adaptations to oxygen depletion occur to make it possible for the hypoxic NRP state to exist. The modified slowly stirred, limited Head Space Ratio (0.5 HSR) method (the Wayne model) was used to investigate the physiological response of MTB to different oxygen tension levels (2). It was shown that abrupt transfer of aerated cultures of MTB to anaerobic condition resulted in their rapid death. The 0.5HSR method involves a slow depletion of oxygen within a sealed, slow stirred culture tube. Initially, cultures are aerobic and the bacteria are grown and replicated by exponential rate. As the available oxygen is gradually depleted, a slowly shift down into a microaerophilic phase and finally into an anaerobic phase is seen. This slow consumption of

oxygen allows the bacilli to adapt and survive under hypoxic NRP state via involving the strategies for restriction of biosynthetic activity, induction of enzyme systems to involve alternative energy sources, and development of mechanisms for stabilizing the bacterial cells and their contents. This would be most favored if essential bacterial cell components were stabilized, minimizing the turnover and the need for energy-expensive synthetic process. Induction of α -crystalline protein and thickening of the cell wall are of the changes occurred while shifting the MTB to hypoxic NRP state. Thus, the process of gradually adaptation to slow depletion of oxygen is so critical for in-vivo and in-vitro survival of MTB. During oxygen shift-down bacterial physiology changes from active growth to a NRP state. Two characteristic stages of NRP are seen; NRP-1 occurs when the oxygen concentration gradually drops to a microaerophilic condition (1% of normal saturation). During this phase, the optical density (OD) of the culture slowly continues to increase due to cell enlargement, and thickening of the cell wall rather than replication which is only seen in hypoxic conditions in MTB. DNA synthesis is terminated, replication of organisms is ceased and RNA synthesis is decreased significantly. A number of enzymatic activities including isocitrate lyase, glycine dehydrogenase and nitrate reductase are known to be increased at this stage (2, 3, 4, 5). The 16 KD α – crystalline protein which plays the most significant role in induction of NRP state in infected human is expressed at the beginning time of NRP-1 stage and is maintained in culture during hypoxic condition (1, 2, 6, 7, 8). The NRP-2 stage occurs when the oxygen concentration drops to anaerobic condition (0.06% of normal saturation); no further increase in optical density is seen. Cell enlargement ceases and the cells become resistant to antibiotics such as isoniazid and sensitive to metronidazole.

Bacteria can persist in NRP-2 stage for a long time. If the NRP-2 state transferred to oxygen-rich fresh medium the bacteria consume oxygen and resume growth in a synchronized replication manner. First, RNA synthesis begins followed by two cycle cell division and then finally DNA replication resumes (2, 7, 8). In this study, the in-vitro induced hypoxically different stages of NRP were set up and the expression of the alpha-crystalline chaperone protein (Acr), which is expressed when MTB undergoes NRP state, was detected. Indeed, the in-vitro activities of rifampin, isoniazid, pyrazinamide, ciprofloxacin and metronidazole were evaluated against two NRP stages of MTB.

MATERIALS AND METHODS

Strain of *Mycobacterium tuberculosis*: All experiments were conducted on clinically isolated MTB strains from the culture collection reserved in the National Reference Tuberculosis laboratory (NRTL) in National Research Institute of Tuberculosis and Lung Disease (NRITLD). Fifty drug susceptible and drug resistant MTB strains were examined in this study.

Culture medium: Dubos Tween-albumin (DTA) medium prepared from Dubos broth base (Sigma), supplemented by albumin V fraction (Sigma) at a final pH=6.6±0.2 according to the manufacturer's instruction. The medium was aseptically dispensed to 20-125 mm sterile screw or rubber-cap culture tubes, (17ml / tube).

Antimicrobial agents: Isoniazid, rifampin, pyrazinamide, ciprofloxacin and metronidazole (all of them were provided from Sigma) were used in 0.4, 0.1, 1, and 1200 µm/ml of concentrations; respectively, at different oxygen tension level conditions (7,8). A special culture medium was used buffered 7H10 broth supplemented with oleic acid dextrose catalase (OADC) at pH=6 to evaluate pyrazinamide effect (9).

Culture for inocula seed preparation: The experimental strains (each experimental set contains 10 strains) of MTB inoculated in to the sterile culture tubes containing DTA and 8 mm long Teflon-coated stirring bar. The inoculated tubes incubated aerobically at 37° C on a magnetic stirring plate with rapid stirring,(at least 250 rpm) to attain an optical density (OD) at 580 nm equal of 0.4. This was corresponded metabolically homogenous to a cell density about 2.5×10⁸ CFU/ml, and was used as working inocula for the slowly stirred, limited head space ratio (0.5 HSR) model of NRP (3, 5, 6, 7).

The 0.5 HSR cultivation method: For the preparation of 0.5 HSR, we used 20-125 mm screw – cap and rubber cap culture tubes (total capacity to the very top lip is 25.5mm), with 8mm long Teflon-coated magnetic stirring bars and 17ml of DTA medium was poured to each tube, leaving an air head space of 8 ml, corresponding to a 0.5 HSR. Each tube was inoculated with 200µl of working inocula (4, 5, 6, 7,10). The inoculated tubes were divided in to four groups for incubation in different oxygen tension conditions as below:

a- A group of inoculated tubes was incubated in aerated condition (the loosely closed screw tubes were incubated at 37° C on a magnetic stirrer at 250rpm).

b- The tightly sealed second group of tubes was incubated at 37° C on a magnetic stirrer at 250 rpm, a speed that produced definite agitation of the surface and promoted rapid and continuous equilibration between headspace available oxygen and medium.

c- The tightly sealed third group of tubes was incubated at 37° C on the biostir magnetic plate at 120 (or the bars spun at a speed sufficient to keep the bacilli suspended throughout the medium but not enough to perturb the surface of the liquid). This group was incubated for expression of the alpha-crystalline protein. Fifty drug susceptible and drug resistant MTB strains were examined in this study.

d- A sufficient number of tubes were tightly closed with a fitted rubber cap, and were incubated at 37° C on the biostir magnetic stirring plate at 120 rpm. This group was incubated for susceptibility assay of the chosen antimicrobial agents against test strains at NRP-1 and NRP-2 stages. Isoniazid, rifampin, pyrazinamide, ciprofloxacin, and metronidazole were added to tubed cultures to yield final concentrations of 0.4, 0.1, 1.0 and 1200 μ g/ml, respectively as follows:

I - Drugs were added to a set of loosely screw capped actively growing aerated cultures when they were in mid-log stage of growth (96 hours old) and aeration continued.

II- A set of slowly stirred 0.5 HSR cultures that were sealed with septum caps receiving isoniazid, rifampin, pyrazinamide and ciprofloxacin by syringe and needle when they were in NRP-1 and at different times during NRP-2.

III- Metronidazole was added to a set of 0.5 HSR cultures immediately after inoculation, since this drug has no effect on actively growing cultures of MTB. Ninety-six hours after addition of isoniazid, rifampin, pyrazinamide and ciprofloxacin, samples were taken for dilution and determination of viability counts (CFU per milliliter).

IV- Drug free 0.5HSR tubed cultures as controls and cultures that contained metronidazole were sampled at the same time as were cultures containing the other three drugs. The counts (CFU per milliliter) of drug added medium were compared with those of drug-free control cultures, and the results were expressed as percent survival compared with these controls (7, 8).

Estimation of oxygen consumption: a sterile solution of methylene blue (500 μ g/ml) was added in some tubed cultures and diluted in the medium to yield a dye concentration of 1.5 μ g/ml. These tubes both inoculated and incubated by test strains for visualization of oxygen consumption, reduction and

decolorization of this dye served as a visual indication of oxygen depletion (7, 11).

Cell disruption & protein extraction: The cultures were heat-killed at 80° C for one hour, consequently the bacterial pellet resulting from centrifugation was washed three times with sterile phosphate buffered saline PBS -T (comprise 0.2% Tween 80) and then twice with sterile PBS . Pellet was re-suspended in cell disruption Tris-HCL (pH=8.8, 15 mM containing 10%, vol / vol of 2 – mercaptoethanol) buffer at an approximate concentration of about 30%(wet wt / vol). An equal volume of 110 micrometer glass beads was added, and sample then was vortexed for about 5 min at high speed (12, 13). Sample was centrifuged at 20000 g (at- 4° C) and the superficial layer carefully transferred to Ependorf tube. The cell-free extracts prepared under these conditions contained about 1 mg /ml of total proteins as determined by the Bio – Rad protein assay Kit..

SDS-PAGE analysis: Twenty-five obtained proteins were mixed with equal volume loading Tris buffer comprise (pH=6.8, 0/064 M containing 2% WT/ VOL SDS, 10% vol/ vol glycerol, 5% vol / vol 2– mercaptoethanol , and 0/0025% bromophenol blue), were denatured in a boiling water bath, then the denatured samples (about 30 μ / well) were fractionated by SDS – PAGE in a Sigma apparatus with a 30% acryl amide concentration of 15% (wt / vol) (13, 14) .

The total protein was stained with 0.25% Coomasse blue, and the results were compared with parallel molecular weight markers that had been purchased from Roche company including bovine serum albumin (67 KD) , catalase (60 KD) , ovalbumin (45 KD) , chymotrypsinogen (25KD) , and cytochrome- C(12.5 KD) (Roche company) . As a whole, 50 drug susceptible and resistant MTB strains were examined for alpha-crystalline protein expression in this study.

RESULTS

Relationship between oxygen consumption and tubercle bacilli adaptation to hypoxic conditions was detected as follows:

1- At periodic intervals the 0.5HSR tightly sealed tubes which were incubated under rapid rotary mixing at 250 rpm, were removed for estimation of the head space O₂ depletion. The rate of headspace O₂ depletion in these tubes paralleled the increase of OD growth at A580 nm, which terminated abruptly at OD equal 0.36. As a result, approximately 1.5 ml (84%) of the originally available O₂ had been consumed (Fig1A). Further depletion of the remaining headspace O₂ continued in these tubes at a slower ratio and was completed after about 35 h of further incubation, while culture turbidity or OD growth remained at plateau situation.

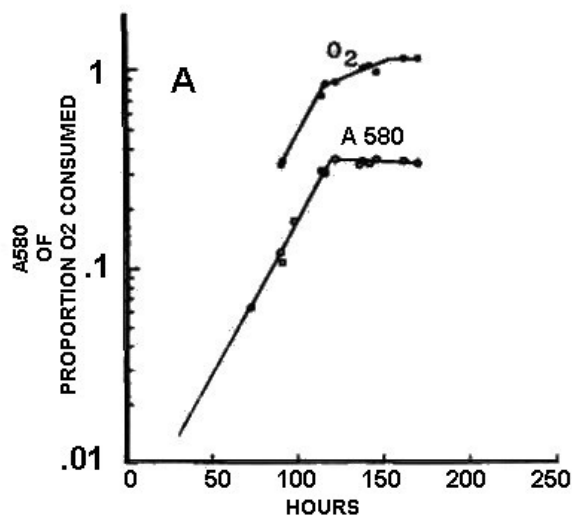


Figure 1 (A): The relationship between the rates of headspace oxygen depletion and growth rates of vigorously agitated.

2- In contrast, in sealed tubes in the second set which were incubated at 120 rpm (slowly stirred), a deflection in the rapid growth curves occurred after less than 72 h, when only 28% of the headspace O₂ had been depleted. Further slow logarithmic

increases of OD at A580 nm were seen for additional periods in excess 100 h before the curves reached plateau (Fig1B).

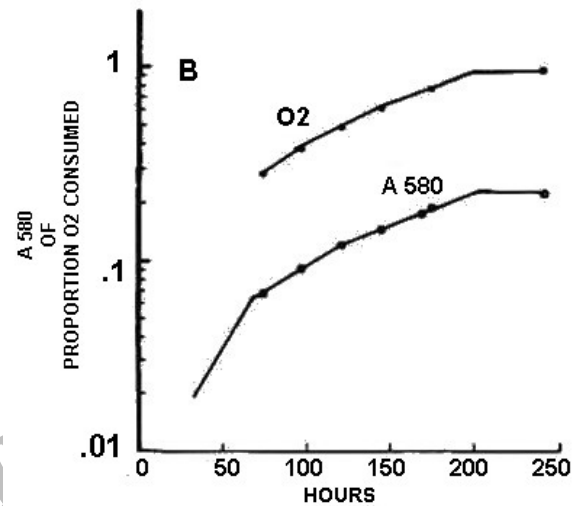


Figure 1 (B). Slowly stirred tubed cultures of MTB at 0.5 HSR. Growth is expressed as log of turbidity (A580) and O₂ depletion is expressed as log of the proportion of initially available headspace consumed.

Some of sealed vigorously agitated and slowly stirred 0.5 HSR tubed cultures were removed at intervals for sampling to assess bacillary CFU count, In tubes subject to rapid rotary mixing, the OD in A580nm readings paralleled the CFU count per ml during the initial growth period (Fig 2A). In parallel cultures that were supplemented with 1.5 µg of methylene blue per ml, visual inspection was indicated that complete decolorization occurred fairly abruptly about 24 h after growth stopped. The viable count did not start to decline notably until about 44 h after the optical increase stopped and then underwent a 55% decline over the 54 h. Thereafter, the CFU count per ml declined with a half life of 100 to 110 h which indicated the partially adaptation to anaerobic condition. With the slowly stirred 0.5HSR tube system, in which the cells remained suspended but the surface was undisturbed and O₂ diffusion was

slow, the rapid replication associated with initially dissolved O₂ lasted about 70 h. Over the next 200 h, the turbidity increased more slowly while the CFU count leveled off, and the slow increase in OD at A580 nm during this stage was not reflected in an increase in CFU count (Fig.2B).

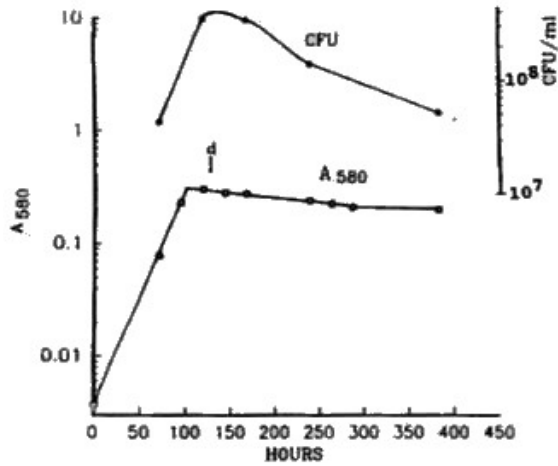


Figure 2(A): Growth, survival, and physiologic states of tubed cultures of MTB grown under 0.5HSR conditions. Turbidimetric assessment (A580) CFU / ml count of cultures growth with rapid stirring.

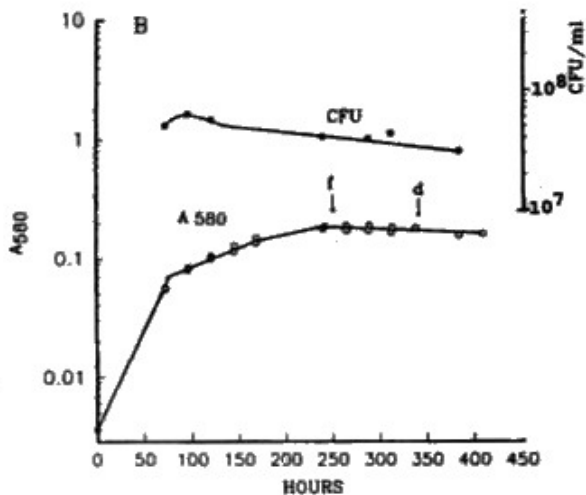


Figure 2(B): Growth, survival, and physiologic states of tubed cultures of MTB grown under 0.5HSR conditions: the corresponding measurement of growth with slow stirring.

However, when a calibrated ocular micrometer was used to estimate the size of the acid – fast

stained bacilli , it was seen that the length of the bacilli increased by about 30% , from a median length of 2.23 μ m during active aerobic growth to 2.94 μ m during the period of O₂ depletion , which is consistent with the increase in turbidity in the absence of replication (Fig.3). The first deflection in the rapid growth was seen before the culture was shifted from active growth into NRP-1 appeared to start about the time that O₂ concentration of the medium approached 1% of the original saturation level (Fig 3).

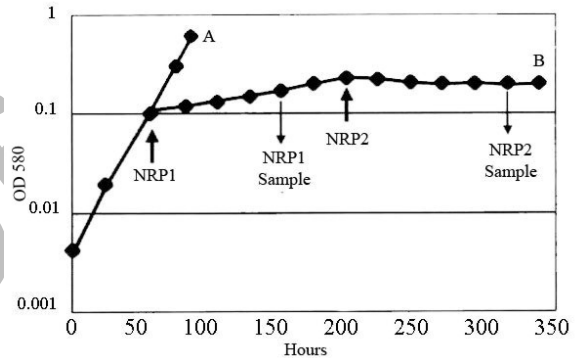


Figure 3. Growth of *M. tuberculosis* in the 0.5HSR slow stirring model of non-replicating persistence. Cultures were set up as described in the methods and growth measured by optical density at 580 nm. Arrows indicate the start of NRP-1 (microaerophilic) and NRP-2 (anaerobic) and the sampling points for markers assessment of each stage. (A) aerobic growth (B) hypoxic culture and NRP growth.

The culture remained in NRP-1 until the O₂ concentration dropped below a level corresponding to approximately 0.06% saturation, when a further deflection occurred as culture entered into NRP - 2 stages and the growth curve showed a plateau.

Isoniazid, rifampin, pyrazinamide and ciprofloxacin killed over 90% of the actively growing aerated drug susceptible test strains within 96 h of exposure.

However, isoniazid and ciprofloxacin had little effects on bacilli in either NRP-1 or NRP-2 stages.

Rifampin exhibited some definite but reduced bactericidal activity against bacilli throughout both nonreplicating stages. Metronidazole did not kill tubercle bacilli in NRP-1 stage and had a little effect early in NRP-2 stages. However, increased killing was seen in later period of NRP-2. It is noteworthy that rifampin continued to exhibit some bactericidal effect even after redox conditions had dropped low enough for the antimicrobial activity of metronidazole to be expressed.

In which stages Acr detection occurred? The Acr protein was detected by both drug sensitive and resistant test groups, but large bands were seen on SDS-PAGE gel in some drug resistant strains. Both drug sensitive and resistant H37Rv strains have been paralleled culture as controls.

Both the tightly sealed tubes that were incubated at 37° C on a magnetic stirrer at 250 rpm (under unlimited O₂), and sealed tubes that were incubated at 37° C on a magnetic stirrer at 120 rpm (slow stirred with limited O₂) as well as the tubes with loosely closed screw that were incubated at 37° C on a magnetic stirrer at 250 rpm (rapid rotary stir) were examined for Acr detection in SDS-PAGE and Acr was detected only in the sealed tubes placed on a magnetic stirrer plate at 120 rpm (slow magnetic stir) in NRP-1 and NRP-2 stages. Acr protein was not detected until aerobic growth shift down in micro aerobic situation was presented. Fifty drug susceptible and resistant MTB strains were examined in this study.

DISCUSSION

When a vigorously aerated culture is abruptly subjected to anaerobic conditions, there is no opportunity for adaptation to occur and unbalanced metabolism leads to rapid death, with a half-life of approximately 10 h (13). However, as shown in the present study, it is possible to regulate the rate of O₂ depletion in a manner that permits the occurrence of

differential adaptive responses which allow the bacilli to survive longer under microaerophilic and/or anaerobic conditions. The pattern of O₂ depletion in a sealed system is determined by both the initial ratio of air to medium and the rate of equilibration between gas and liquid phases (Fig.1B). In rapidly agitated tubes on a stirrer plate at 250 rpm, equilibration between gas and culture medium is so efficient that no deflection in the growth curve is seen until the head space O₂ has been about 80% depleted. However, depletion of the balance of the O₂ thereafter is so rapid that growth terminates abruptly (Fig.1A), and only a minimal adaptation to anaerobic conditions occurs; a decline in CFU, is seen, with a half-life of about 54 h (Fig.2A). Further declines in CFU are seen with a half-life of around 100 h, suggesting partial adaptation of the survivors to a greater tolerance to O₂-limited conditions. In contrast to the rapid depletion of O₂ in vigorously rotated cultures, the cultures subjected to slow magnetic stirring at 120 rpm with no detectable perturbation of the surface of the medium had a much slower rate of equilibration with the air, and the head-space exhibited a much slower rate of O₂ depletion. When the O₂ content of the head space air was reduced by only 30%, an abrupt deflection in the turbidity curves occurred, there after culture underwent into NRP-1 stage and the turbidity increase was continued but reduced until the O₂ was gone approximately 0.06% and the slowly increase in turbidity ceased. As shown in Fig-2 the growth curve is plateau. Unexpectedly, the stage of slow increase in turbidity was not reflected by an increase in CFU (Fig.2B). This period during which turbidity increased slowly without significant change in CFU counts is perhaps the most interesting as a model for studying the ability of tubercle bacilli to survive under conditions that do not support replication, since it probably resembles conditions encountered in inflammatory or necrotic tissue. Expression of Acr was also initiated at this

stage. The termination of DNA synthesis (Fig. 3) coincided with the first deflection in the turbidity curve and the cessation in increase in CFU counts, confirming that replication had indeed terminated rather than reflecting a balance between replication and death of cells. Further insight into the individual nature of the bacilli in NRP-2 stage under complete O₂ depletion is offered by the fact that bacilli exhibited synchronous replication upon resumption of aeration. There must be a well-defined induction of a highly specialized array of regulatory products which regulate synthesis of some enzymes that are involved in maintenance of metabolism, while repressing the production or activity of others that are involved in cell replication as O₂ is depleted and a reversal of these functions when aeration is resumed. Studies of these mechanisms are presently under investigation in many laboratories (9,15).

The experiments involving the use of antimicrobial agents provide additional insights about the phenomenon of non-replicating persistence. Isoniazid is believed to exert its bactericidal effect by interfering with synthesis of mycolic acids which are needed for the cell wall biosynthesis (10); there is an evidence that this effect depends on interaction of the drug with mycobacterial peroxidase and H₂O₂ to produce reactive oxygen radicals (16). Our observations regarding that enlargement of bacilli occur without replication during NRP stage 1 suggest that some component of cell wall synthesis was continued in an alternating form. The failure of isoniazid to exert pronounced bactericidal effects under these conditions on isoniazid susceptible strains probably reflects the suppressed production of H₂O₂ during the microaerophilic metabolism. Ciprofloxacin inhibits the A subunit of DNA gyrase (17), which is essential for DNA synthesis, and the failure of this drug to exert bactericidal effects on MTB that are not replicating or producing DNA is consistent with its mechanism. Rifampin, on the

other hand, inhibits DNA-dependent RNA polymerase (18) and continued to exhibit some bactericidal action even after O₂ depletion occurred in NRP-1 stages. The demonstration of some continuing action of rifampin on bacilli in these stages, even after the redox potential has dropped sufficiently for metronidazole to initiate its bactericidal action, supports this fact that the enzymes which may be induced during microaerophilic, or even subsequent anaerobic conditions for alternating metabolism are essential to survival of the organism. Metronidazole exerts its bactericidal action through an intermediate reduction product that is produced only at a reduction potential below -430 mV which kills by nicking DNA (5, 7, 8). The identity of an enzyme system in MTB that is capable of producing this low reduction potential remains to be established.

Since almost all TB patients have antibody to the Acr protein of MTB, it is ubiquitous in infected individuals. Since inflammatory, granulomatous and necrotic lesions have hypoxic conditions, the hypoxic shift down to NRP1 stage seen with this organism in the in-vitro study suggests that this protein is expressed in the human host as well. The induction of Acr production during hypoxic shift down, suggests a critical role for this protein in the ability of MTB to persist without replicating in the hostile regions of the host tissues. On the other hand, the 16-kDa α -crystalline protein of MTB might represent a good antigenic target of protective T cell responses. In fact, this protein is predominantly expressed by MTB during stationary growth or subjected to oxygen deprivation in NRP stages, and can account for up to 25% of total bacillary protein expressions in these circumstances. Increased expression of the gene encoding the 16-kDa protein has been detected in *M. tuberculosis*-infected mice and in lung biopsy specimens from tuberculosis patients with chronic cavitory lesions. As MTB being confined within the

granuloma may induce similar conditions to those mentioned above. The 16-kDa protein might be an important antigenic target during bacillary latency (19). The peptide p91–110 of the 16-kDa protein has been widely studied and is well known for its properties of immunodominance and genetically permissiveness, representing a candidate subunit vaccine component (6,12). Additionally, it was recently shown that fine specificity, clonal composition and HLA-II restriction of CD4 T cells are cloned specifically for the P 90-110 epitope of 16 KD α -crystalline protein(20,21,). Post-chemotherapy changes in T cell proliferation and IFN- γ responses in TB patients are particularly striking for T cells recognizing the 16-kDa antigen and its peptide p 91–110 which causes strong induction in binding to all tested HLA-DR molecules. Therefore, understanding the mechanisms of induction of factors associated with the hypoxic condition of tubercle bacilli especially α -crystalline protein should contribute to the development of strategies for preventing that persistence in human lesions.

A slow depletion of O₂ appears to permit the occurrence of adaptations that favor long-term non replicating persistence of tubercle bacilli under microaerophilic conditions and also enhance the ability of the bacilli to survive in anaerobic conditions. This versatility could account for long-term latency of tuberculosis in the human host. Much effort today is directed toward characterizing the interaction of tubercle bacilli with various components of the host cellular defense mechanism, and this is critical for understanding the process of early infection and induction of disease. However, bacilli also persist in necrotic areas of host tissue which may be acellular and /or vascular and are ; thus, exposed to a range of physiologic conditions. The model presented here may be useful for identifying the molecular events including mRNA expression and native products such as α -crystalline

protein and others that are responsible for the versatility of MTB to survive under a range of conditions, as well as those associated with reactivation of these bacilli.

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