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Review

Dormancy models for *Mycobacterium tuberculosis*: A minireview

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Abstract

Dormancy models for *Mycobacterium tuberculosis* play important roles in understanding various aspects of tuberculosis pathogenesis and in the testing of novel therapeutic regimens. By simulating the latent tuberculosis infection, in which the bacteria exist in a non-replicative state, the models demonstrate reduced susceptibility to antimycobacterial agents. This minireview outlines the models available for simulating latent tuberculosis both *in vitro* and in several animal species. Additionally, this minireview discusses the advantages and disadvantages of these models for investigating the bacterial subpopulations and susceptibilities to sterilization by various antituberculosis drugs.

Key words: dormancy, latency, tuberculosis, stationary-phase.

Introduction

An important part of tuberculosis pathogenesis is the latency stage, in which maintenance of the asymptomatic state depends on containment by the host immune response (Stead *et al.*, 1968; Wayne and Sohaskey, 2001). Approximately one-third of the global population is infected with *M. tuberculosis* that exists in the latent stage, with reactivation occurring in 2-23% of cases (Stead *et al.*, 1968; Wayne and Sohaskey, 2001). There is widespread confusion regarding the terms describing this physiological phenomenon of *M. tuberculosis* (Kell and Young, 2000). Authors may use the terms "dormant," "latent," or "persistent" interchangeably. By definition, latency is a clinical condition referring to an organism persisting for many years within the host without causing clinical disease or showing reactivity in a tuberculin test. The dormant bacilli are characterized by slow *in vitro* growth, a downshift of metabolic pathways, altered staining features, an inability to be cultivated on solid media, and resistance to antimycobacterial agents. Wayne and Sohaskey refer to the latent physiological state as "non-replicating persistence" (NRP) when the bacilli evade the host immune response and survive inside the hostile macrophage environment (Stead *et al.*, 1968; Wayne and Sohaskey, 2001). The early work of Mitchison and Dickinson set the basis for the hypothesis that four bacterial subpopulations can coexist in a tuberculous lesion – Figure 1 (Dickinson and Mitchison, 1970; Dickinson and Mitchison, 1966). It is believed that a typical tuberculosis lesion is composed of bacteria at different physiological states. The most actively dividing bacteria are killed at the beginning of tuberculosis therapy and prolonged treatment is required to eliminate the non-replicating subpopulations that persist due to reduced susceptibility to standard antituberculosis agents (Hu *et al.*, 2003b).

Grosset's model regarding the subpopulations of *M. tuberculosis* divides the bacilli into three groups. The first population represents the bacilli that are actively dividing at neutral pH in the liquefied caseous part of a two square-centimeter pulmonary cavity (approximately 10^8 organisms); the second group represents the intracellular mycobacteria that grow slowly and reside in the macrophages (approximately 10^5 organisms). The last population represents the slowly growing *M. tuberculosis* (approximately 10^5 organisms) (Grosset, 1980). Another hypothesis regarding the different populations present in tuberculosis disease was developed by Gillespie and McHugh; in this hypothesis, the bacteria are postulated to exist within three compartments: those existing in the bronchioles and alveoli, those in the cavitary lesions or empyema, and those bacilli which, in an acidic, intracellular medium, pyrazinamide exhibits the most powerful anti-tuberculous action (Gillespie, 2002). In this model, it is assumed that an effec-

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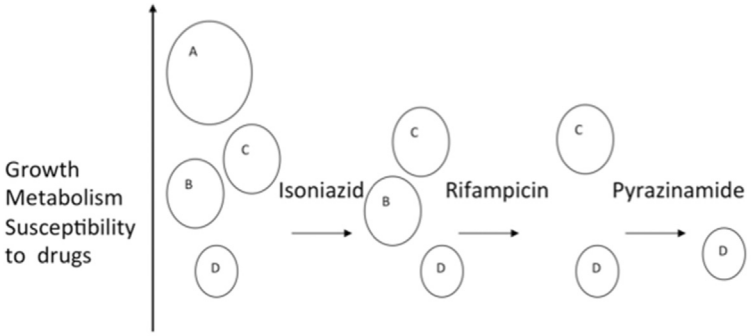

Figure 1 - The bacillary four-population theory: A) isoniazid-susceptible population, B) rifampicin-sensitive population, C) pyrazinamide-susceptible bacilli in acidic pH, and D) non-replicating persisters that are not killed by high doses of rifampicin.

tive cure relies on the degree of drug penetration. The size of cavitations has been linked to the treatment outcome but there is not yet experimental evidence showing the penetration level in the pulmonary cavities. Several *in vitro* and animal models have been proposed to study the NRP of *M. tuberculosis*. Although none of these models truly represents human latent tuberculosis (Stead *et al.*, 1968; Wayne and Sohaskey, 2001), the models have great potential to contribute to a better understanding of the latent tuberculosis infection. The minireview addresses the *in vivo* and *in vitro* models that are used to simulate latent tuberculosis.

Animal Models

Various animal models have been used to study the dormancy of *M. tuberculosis* in humans. Mice have commonly been used because of the convenience, the logarithmic expansion of mouse genetic experiments, together with the technical benefits, such as the availability of various strains and low-cost reagents. However, tuberculosis in the mouse model differs from the human disease in several important aspects; particularly, bacillary burden can be very high in susceptible mice and is not ultimately cleared by the host (Cosma *et al.*, 2003). Moreover, the immune response in the mouse, in the form of granuloma, differs from that in humans as no caseation or calcification occurs (Sugawara *et al.*, 2004). Other researchers have used rats instead of mice, as rat tuberculosis is thought to be more representative of human tuberculosis (Sugawara *et al.*, 2004; Elwood *et al.*, 2007). Guinea pigs exhibit certain pathological features similar to those observed in humans; however, unlike humans, guinea pigs are extremely susceptible to primary pulmonary disease progression, making them more suitable for tuberculosis vaccine models. Rabbits display a range of pathological features in common with humans, ranging from spontaneous healing to caseous and cavitary pulmonary lesions. The main limitation with rabbit models is the natural resistance to *M. tuberculosis* infection and susceptibility only to *M. bovis* infection. Non-human primates were also used as models for *M. tuberculosis* and showed variable pathology aspects, some of which were indistinguishable from human tuberculosis. A promising model is the cynomolgus macaques model, which allows the study of the interaction between an HIV-like virus (Simian immunodeficiency virus, SIV) and *M. tuberculosis* in a natural host, thus functioning as a model of tuberculosis and AIDS co-infection (Flynn, 2006). *M. marinum* is a natural fish pathogen that was used by several researchers as a model of human tuberculosis; it causes granulomas with caseous necrosis (Flynn, 2006). Interestingly, the optically transparent zebrafish model developed by Ramakrishnan's group shows caseating granulomas upon infection with *M. marinum* (Swaim *et al.*, 2006). In this model, it was shown that zebrafish lesions, in contrast to human tuberculous granulomas, tend to contain few lymphocytes. The zebrafish model simulates several aspects of human disease, such as the ability to establish either an acute or chronic infection based upon the initial inoculum. Additionally, the progression of necrosis to thin-walled cavities mimics the pathological events that occur in human tuberculosis. A recent paper highlighted the potential role of medaka as an aquatic model in exploring various aspects of TB latent infection (Broussard and Ennis, 2007). The authors compared medaka and zebrafish infected with *M. marinum* and concluded that both serve as a useful dual-model. Specifically, the zebrafish model may better represent the acute phase, while the medaka model provides insight into latency and chronic infection. Animal models other than mice better simulate human disease but are limited by low availability, space requirements, handling difficulties, cost, genetic variability, and lack of available reagents. The two most commonly used models to represent latent tuberculosis are discussed in the following section.

The Cornell Model (The Treated Mouse Model)

The Cornell model of dormancy, developed by McCune and colleagues at Cornell University in the 1950s, provided the first experimental *in vivo* evidence of the existence of latent bacilli (McCune *et al.*, 1966). In this model, mice are infected intravenously with a high dose of virulent *M. tuberculosis* for an extended period and then treated with antimycobacterial drugs to reduce the bacterial burden

to undetectable levels. Although no viable mycobacteria are detected immediately following treatment, reactivation of the infection can occur spontaneously, a condition referred to as pseudosterilization, or in response to immunosuppressive agents, such as corticosteroids (McCune *et al.*, 1966). Bacteria recovered from the Cornell model are known to be drug susceptible. This model is attractive because the low bacterial burden on the mice is similar to paucibacillary latent human tuberculosis. However, introduction of antibiotics to induce reduction in mycobacterial load does not mimic the natural pathogenic introduction of tuberculosis, making it a debatable microbiological model for latency and immunological investigation.

The Low Dose, Chronic Murine Model (The Untreated Mouse Model)

In the untreated mouse model, a low dose of *M. tuberculosis* infection delivered via the aerosol or intravenous route results in a slow but steady increase in bacterial numbers in the lungs. After an initial acute phase of bacterial multiplication, infection is contained solely by the host immune response resembling latency in humans. The plateau level of the bacillary count is proportional to the original inoculum size (Sever and Youmans, 1957). Unlike latent tuberculosis in humans, this model results in a high bacillary burden, and the animals eventually die due to lung pathology caused by inflammatory response. Although this approach does not mirror true latency, this model of a persistent infection state has been used for its simplicity and induction of prolonged chronic tuberculosis (Stead *et al.*, 1968: Wayne and Sohaskey, 2001).

In vitro Models

Most dormancy models are based on gradual depletion of oxygen upon long incubation, a state that reflects human disease where poor aeration is a characteristic of the calcified granulomas. Other stresses have been applied to induce the persistence state of *M. tuberculosis,* such as nitrous oxide treatment or nutrient deprivation (Sever and Youmans, 1957; Betts *et al.*, 2002; Wayne and Sohaskey, 2001). The first attempt to mimic dormancy was performed by Corper and Cohn in the 1920s who kept several sealed cultures of human isolates at 37 °C for 12 years and were able to isolate 0.01% viable organisms from the sediment of the bottles (Parrish *et al.*, 1998). Subsequently, several dormancy models have been proposed (Table 1). Some of these models are presented in the following section.

The Wayne hypoxia model

In the Wayne hypoxia model, the bacilli are exposed to gradual hypoxia (Wayne and Hayes, 1996). In the original unstirred Wayne model, cultures were not shaken during incubation in glycerol-free medium. Equilibrium separation was established by the settling of the bacilli through a self-generated oxygen gradient between the anaerobic bottom of the tube and the replication of bacilli in the upper, oxygen-rich region of the tube (Wayne and Hayes, 1996; Wayne and Sohaskey, 2001). This allows the bacilli to

Table 1 - Commonly used *in vitro* dormancy models for *Mycobacterium tuberculosis*.

Model	Principle	Reference
Corper and Cohn experiment	Adaptation to stationary phase in conventional culture	(Parrish *et al*., 1998)
Mitchison and Dickinson	Intermittent incubation at ranges of temperatures	(Hu *et al*., 2000; Hu *et al*., 2003a)
Wayne model	Gradual oxygen depletion in sealed culture tubes with agitation	(Wayne and Hayes, 1996)
Hundred-day static culture	Prolonged cultures in stationary phase without agitation and generation of rifampicin tolerance	(Hu *et al*., 2006)
Starvation model	Cultivation in a nutrient-low medium	(Betts *et al*., 2002)
Chemostat culture system	Growth under defined conditions and adaptation to static culture through carbon starvation	(James *et al*., 2002)
In vitro granuloma model	Mycobacterium bovis BCG strain carrying a luciferase (*lux*) gene and lung myofibroblasts mixed with fresh peripheral blood mononuclear cells to form a granuloma	(Puissegur *et al*., 2004)
Nitrous oxide-based model	Exposure to low-dose nitrous oxide	(Voskuil *et al*., 2003)
Hypoxic resazurin reduction assay	Culture aliquots in vacutainer tubes followed by adding of redox indicator and visual inspection	(Taneja and Tyagi, 2007)
Low-oxygen recovery assay (LORA)	Recombinant H37Rv expressing a dormancy luciferase gene from *Vibrio harveyii*	(Cho *et al*., 2007)
Whole-cell nitrate reductase assay	Development of Wayne model in microplate format and monitoring nitrate reductase activity	(Khan and Sarkar, 2008)
Microplate Phosphorylation Assay for the regulatory 2-component system DevR-DevS/Rv2027c	96-well format and analysis by gel electrophoresis or radioactivity measurement by scintillation counting	(Saini and Tyagi, 2005)

adapt to the early microaerophilic state and the later anaerobiosis with a half-life tenfold greater than that of bacilli suddenly deprived of oxygen. In the improved version of the model, the cultures are incubated in a limited headspace ratio and subjected to slow magnetic stirring to control the rate of oxygen consumption. This design favors the occurrence of dormancy events along a temporal rather than a spatial gradient. Although this is an *in vitro* dormancy model, it is thought to simulate phenotypic alterations of latency observed *in vivo* and correlate with the anaerobic condition seen in human infection. However, the main limitation of the model is the lack of standardization; many experimental variations exist, making comparative studies difficult. Additionally, the Wayne model does not explain other aspects of dormancy, such as bacterial adaptation to other stresses encountered during dormancy. The Wayne model can also be criticized regarding the high viability of the bacilli recovered from it. Another shortcoming of the model is the susceptibility to metronidazole, which has been shown to contradict with the *in vivo* findings (Wayne and Sohaskey, 2001; Klinkenberg *et al.*, 2008). Nevertheless, the Wayne model represents a valuable and informative method that has contributed to the understanding of the bacillary dormancy and the metabolic adaptation of the organism during the stationary phase.

The Loebel nutrient starvation model

Betts *et al*. used a modified Loebel nutrient starvation model to investigate the effect of granulomas on the metabolism of *M. tuberculosis* (Loebel *et al.*, 1933; Betts *et al.*, 2002). The model is based on the assumption that *M. tuberculosis* resides in tissues where nutrients and other essential factors are likely to be limited. Cultures were transferred from nutrient-rich medium into phosphate-buffered saline, resulting in a gradual shutdown of respiration to minimal levels, but the bacilli remained viable and recovered when re-inoculated in rich medium. *M. tuberculosis* isolated from lung lesions has been found to display altered colonial morphology with cell wall thickening and loss of staining properties similar to those isolated from pulmonary lesions (Hu *et al.*, 2003a). At the genetic level, the bacilli exhibit a global down-regulation in gene expression (Betts *et al.*, 2002). Importantly, the bacillus remained resistant to metronidazole, unlike the case for the Wayne model.

The 100-day static culture model of dormancy

A development of the population theory (Figure 1) is the hundred-day static culture system, patented by Hu and Coates (Hu *et al.*, 2006). This model represents the coexistence of four bacterial subpopulations in a tuberculous lesion (Hu *et al.*, 2003a). The basic premise is a gradual depletion of nutrients and self-generated hypoxia upon incubating the cultures for a long time without agitation. Following the prolonged period of incubation, the cultures are resuscitated by transferring them into fresh broth, and then a portion is exposed to a high concentration of rifampicin to induce phenotypic tolerance resembling the two less susceptible subpopulations in human tuberculosis (Hu *et al.*, 2003a). Relapse of drug-susceptible tuberculosis occurs in up to 5% of successfully treated patients and is thought to be the outcome when the dormant population is not substantially reduced (Hu *et al.*, 2003a; Stead *et al.*, 1968). The existence of this population has been supported by demonstrating the transcriptional activity of dormant cells in the drug-treated murine model. The transcriptional activity indicates that the dormant bacilli are metabolically active, which raises the possibility of finding novel antituberculous drugs with activity against this dormant population (Hu *et al.*, 2000). The application of the model to test pyrazinamide showed that the drug is effective at killing semi-dormant bacilli in acidic conditions but not the resuscitated, phenotypically resistant bacteria. Thus, the new model is a useful tool to search for sterilizing drugs. Technical limitations need to be considered as possible sources of bias in the conclusions drawn from this model, particularly clump formation. Clumping interferes with all studies investigating mycobacteria, especially complicating dormancy experimental work following a long period of incubation. Water bath sonication is used to disintegrate clumps, followed by homogenization of samples by re-suspending harvested bacteria in fresh broth containing the detergent Tween-80 to inhibit further clumping. However, the effectiveness of sonication in disrupting clumps of mycobacteria is variable (Al-Sayyed *et al.*, 2007; Stokes *et al.*, 2004), and prolonged sonication is not recommended because of its adverse effects on the viability of mycobacteria (Al-Sayyed *et al.*, 2007; Stokes *et al.*, 2004). Other mechanical measures that may help overcome the high variability in colony count include agitation with glass beads. However, this procedure is generally avoided for safety reasons and to avoid potentially altering the mycobacterial surface, which could affect the interaction with the antimycobacterial compounds (Al-Sayyed *et al.*, 2007; Stokes *et al.*, 2004). The design of the 100-day culture model would be simplified by adaptation for use in microvolumes to allow screening for new sterilizing drugs. The method might also be improved by starting with a lower inoculum to reduce clumping as well as manual agitation of the cultures to achieve consistent colony counts.

Controlled batch culture model

A special form of chemostat culture system, the controlled batch culture, has been proposed as a dormancy model, which offers a well-controlled environment with precise levels of nutrients, oxygen and pH (James *et al.*, 2002). Other favorable features of this model are the ability to handle large volumes of cultures, the high colony count maintained after 100 days (10^6–10^7 cfu/mL), and the capacity for time-point sampling. However, this instrument is not widely available, and little is known about its predictive

value for known anti-tuberculosis drugs. Another drawback of this model is the need for large amounts of the compound to screen novel drugs, which is not always feasible.

Hypoxic resazurin reduction assay

A recently published model of dormancy is the hypoxic resazurin reduction assay, which relies on color changes of the redox indicator resazurin upon exposure to viable bacteria (Taneja and Tyagi, 2007). A small volume of culture is incubated in this model for four days and visually read on the fifth day after 24 hours incubation with resazurin. The system is convenient in terms of the small volumes (and thus reduced drug quantities) needed for testing. Fast screening is also possible as only compounds that show preliminary anti-dormancy activity would be processed for colony counts on solid media. Despite its technical advantages, limited data are currently available from this model regarding its predictive value, as pyrazinamide, the main sterilizing agent, has not yet been tested in this model.

Other in vitro models of dormancy

Other models of dormancy have been described but considerable expertise is required to operate them (Table 1). It is difficult to extrapolate which model is most likely to mimic the genuine mycobacteria persistent condition, and these different models might represent different metabolic states at various stages of infection. For example, the stationary phase culture is similar to the bacilli during latent infection, while the 100-day culture model mimics the persistent bacteria following chemotherapy. Hypoxia-based models are thought to be more representative because of the induction of several responses that are characteristic of human disease and absent in other models of dormancy, *e.g.*, acr is overexpressed only in response to hypoxia. Antibodies to this chaperone protein are abundant in latent infection (Wayne and Sohaskey, 2001).

Dormancy models and the need for sterilizing drugs

Treatment of active tuberculosis requires a lengthy regimen with multiple drugs to eradicate the infection, regardless of culture conversion within a few weeks. This complicated regimen leads to poor adherence of patients to the treatment protocol and subsequent development of drug resistance. Therefore, a major goal in the field of tuberculosis drug development is shortening the course of therapy by finding drugs with powerful activity against dormant bacilli. A compound with sterilizing power is a valuable candidate for intensive investigation and development. Such a drug will not only shorten the treatment course but also contribute to treating the latently infected population worldwide. Thus, assessment of novel anti-tuberculosis leads should include early screening of their activity against dormant bacilli to identify valuable agents. The models of tuberculosis dormancy aid in testing new drugs against simulated persistent tubercle bacilli and in the design of an effective short anti-tuberculosis regimen.

The persistent bacilli create a significant challenge for effective tuberculosis therapy, as conventional treatment is mainly active against replicating bacilli. This is not unexpected, as conventional anti-tuberculosis drugs were developed by screening molecules against actively growing, log-phase bacilli. Eradication of persistent bacilli will allow shortening of treatment. The ability of a drug to kill persistent organisms that possess limited metabolic activity is referred to as “sterilizing” activity. The term was first used to describe the action of pyrazinamide in the mouse model of tuberculosis (McCune *et al.*, 1956). In animal models of tuberculosis, isoniazid has relatively poor sterilizing activity while pyrazinamide and rifampicin can render organs sterile (McCune *et al.*, 1956). Although isoniazid is a poor sterilizing agent, it dramatically reduces the rate of reactivation among tuberculin skin test-positive individuals, demonstrating that bacteria retain some replicative capacity as the susceptibility to isoniazid requires some level of metabolic activity and cell division (Comstock *et al.*, 1979). This result is also supported by the finding of mRNA expression in dormant tubercle bacilli (Hu *et al.*, 2000). Rifampicin and pyrazinamide are the two drugs with the greatest activity against dormant *M. tuberculosis* (Hu *et al.*, 2003a). The introduction of these two agents shortened the treatment course from eighteen to six months. High dose rifampicin kills most non-actively replicating bacteria with a small proportion of the bacilli persisting (Hu *et al.*, 2003a). Although Hu *et al.* have proven that the dormant bacilli have active transcriptional activity even when cell division is arrested, a proportion of those persisters are not killed by any of the known antimicrobial drugs and can resist even high doses of rifampicin *in vitro* (Hu *et al.*, 2006). Among the clinically tested drugs, the fluoroquinolones, such as moxifloxacin and the nitroimidazopyran PA-824, show significant sterilizing power (Hu *et al.*, 2008). Moxifloxacin is currently undergoing a phase-three FDA-approved clinical trial (NCT00864383) for shortening the treatment course to four months by replacing ethambutol (Ginsberg and Spigelman, 2007). PA-824 showed great sterilizing activity *in vitro* and in mice models (Tyagi *et al.*, 2005). The dormancy-induced genes can serve as tools for designing anti-dormancy drugs with specific sterilizing power. In addition, the *in vitro* models of dormancy play an important predictive role in the design of any novel therapeutic regimen despite the discrepancies that might occur in some cases (Wayne and Sohaskey, 2001; Klinkenberg *et al.*, 2008). Drugs that show promising minimal inhibitory concentrations against log phase cultures need to be tested in a simple *in vitro* model of dormancy during early screening to ensure the drug has properties that meeting one of the Millennium Development Goals set by the World Health Organization, ultimately shortening tuberculosis therapy (Glaziou *et al.*, 2013).

Concluding Remarks

The mechanisms by which the tubercle bacilli survive in a dormant state inside the human host is not well understood. This lack of understanding is a main obstacle to modeling and studying latent and persistent tuberculosis infection. Continuous efforts are needed to develop improved *in vivo* and *in vitro* models of persistent *M. tuberculosis* in order to have a high predictive sterilizing index. Achieving these goals is crucial for meeting the global targets of fighting tuberculosis through new, effective, and shorter regimens that show powerful sterilizing properties in the dormancy models. In addition, these models will support the preventive therapy in latently infected populations.

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