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Review

Understanding anti-tuberculosis drug efficacy: rethinking bacterial populations and how we model them

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ABSTRACT

Tuberculosis still remains a global health emergency, claiming 1.5 million lives in 2013. The bacterium responsible for this disease, *Mycobacterium tuberculosis* (*M.tb*), has successfully survived within hostile host environments, adapting to immune defence mechanisms, for centuries. This has resulted in a disease that is challenging to treat, requiring lengthy chemotherapy with multi-drug regimens. One explanation for this difficulty in eliminating *M.tb* bacilli *in vivo* is the disparate action of antimicrobials on heterogeneous populations of *M.tb*, where mycobacterial physiological state may influence drug efficacy. In order to develop improved drug combinations that effectively target diverse mycobacterial phenotypes, it is important to understand how such subpopulations of *M.tb* are formed during human infection. We review here the *in vitro* and *in vivo* systems used to model *M.tb* subpopulations that may persist during drug therapy, and offer aspirations for future research in this field.

1. Pathogenesis of *Mycobacterium tuberculosis* – an overview

Tuberculosis, a disease caused by *Mycobacterium tuberculosis*, is primarily transmitted through the respiratory route. Individuals become infected when they inhale aerolised particles produced by patients with active disease. These droplet nuclei (measuring ~1-3 μm and containing 1-3 bacilli) are then engulfed by alveolar macrophages, where *M. tuberculosis* (*M.tb*) bacilli are able to evade killing and continue to multiply by avoiding phagosome-lysosome fusion.[1] Additional macrophages and other immune cells then become localised to the site of infection creating an ordered cellular architecture known as a granuloma. These dynamic structures evolve from simple cellular aggregations with vascular elements to necrotic lesions characterised by hypoxia and nutrient deprivation.[2,3] Caseous necrosis often ensues; this consists of the "solid" necrosis of the exudative lesion and some of the lung tissue that surrounds it. The process likely results in the death of the majority of *M.tb* bacilli but some will survive extracellularly in the solid caseous material. Caseous necrosis can result in the abolishment of the neighbouring host tissues and if this destruction reaches the bronchiolar barrier then a cavity is formed, creating a route of dissemination for *M.tb* bacilli through the airways.[4] The *M.tb* load in tuberculous cavities may reach 10^{11} CFUs (Colony Forming Units) per gram, with bacilli presumably replicating rapidly in this environment.[5] Thus patients with cavitation are highly infectious.[6] Furthermore, the degree of cavitation is often one of the only factors associated with treatment failure.[7] Thus, *M.tb* bacilli are able to survive in multiple diverse and dynamic environments during infection; drug regimens that are able to kill bacilli in all these niches are likely to offer the best opportunity to reduce treatment length and eliminate relapse.

2. *M. tuberculosis* populations within the host

The pathogenesis of *M. tuberculosis* creates bacterial phenotypic heterogeneity, defined here as a mixture of genetically identical bacteria that vary in measured characteristic(s). This heterogeneity may impact upon the metabolic state of *M.tb* and/or the efficacy of antimicrobials. Thus *M.tb* infection rather resembles, and might be approached as, a polymicrobial infection where several cidal activities are required for *M.tb* sterilisation by chemotherapy.

When exposed to bactericidal concentrations of antimicrobial drugs, the number of viable cells in a susceptible bacterial population does not decline exponentially. Instead, the mortality rate decreases over time and a substantial fraction of the population may survive antimicrobial drug treatment. This

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phenomenon has been observed for virtually all antimicrobials used in clinical practice and for many bacterial species[8–14] and has been attributed to antimicrobial tolerance. Phenotypic antimicrobial tolerance is a temporary, reversible bacterial state that is often associated with a reduced rate of multiplication, where some antimicrobial drugs are ineffective against genetically susceptible bacilli. Antimicrobial tolerance is hypothesised to be the prime reason for the extended treatment regimens required for *M.tb* chemotherapy, as fully drug-sensitive bacilli survive (persist through) initial antimicrobial drug therapy.[15–17] It has long been speculated that *M.tb* in a non/slowly-replicating state may play a clinically significant role, persisting during drug therapy.[18–20] The first-line antimicrobials used to treat *M.tb* infection (isoniazid, rifampicin, pyrazinamide and ethambutol) are all active against actively-replicating bacteria,[21] however effectiveness of this drug regimen against non/slowly-replicating bacilli is reduced or eliminated.[22–24] This *M.tb* slow/non-replicating state is hypothesised to be induced by the environmental conditions found in specific granuloma types, in particular those associated with hypoxia or nitric oxide production.[18,25,26] An *M.tb* transcriptional signature resembling slow or non-replicating bacilli was also identified in bacilli isolated from human sputa.[27] Exposure of bacilli to such microenvironments results in the expression of a discrete set of genes known as the dormancy regulon (DosR/DevR) that are in turn responsible for transitioning bacilli into a non-replicating and hence likely drug-tolerant state.[25,28-30] The mechanism(s) that result in the generation of phenotypic drug tolerant *M.tb* populations *in vivo* are currently not well understood, however it is critical to consider these sub-populations of bacilli for the development of more effective drug regimes.

The *M.tb* population *in vivo* has been compared to a Russian nesting doll, consisting of layer after layer of distinct bacterial subpopulations that may also be separated by time and space, each of which may be differentially killed by various antimicrobial drugs dependent on phenotypic drug tolerance or anatomical location. The models developed by Mitchison[31] and Mitchison and Coates[17,32] are often adapted to describe four populations defined by antimicrobial drug efficacy (1) actively growing bacilli mostly killed by isoniazid, (2) slow/non-replicating *M.tb* bacteria that undergo spurts of metabolism, which are killed by rifampicin, (3) intracellular bacilli present in the acidic compartments of macrophages or in acidic lung lesions that are killed by pyrazinamide, and (4) *M.tb* persisters found in hypoxic microenvironments with much reduced action of most anti-TB drugs.[22,33] As evidenced in TB patients that relapse during treatment of drug-sensitive *M.tb*, the host immune system cannot effectively eliminate these residual *M.tb* bacilli that are not killed by chemotherapy. Therefore, although achieving a clinical cure, the current anti-TB standard regimen does not necessarily achieve a bacteriological cure. In other words, current therapy does not completely eradicate all bacilli from the body, but allows the infection to be contained effectively for long-periods of time.[34] These observations underscore the need for developing better sterilising compounds against *M.tb*. However, the lack of adequate screening systems able to identify new compounds effective against drug-tolerant *M.tb* phenotypes remains an immense barrier to the anti-tuberculosis drug development process.[35]

3. Models to study *M. tuberculosis* populations that may persist during drug therapy

3.1. In vitro studies

Several models have been developed to recreate conditions encountered by *M.tb* within the host during infection. Since the nomenclature surrounding *M.tb* dormancy and latency is ambiguous, we refer to these models here simply as systems that generate populations of bacilli that may persist (or at least be differentially killed) by antimicrobials during disease. The *in vitro* systems necessarily only capture specific aspects of the clinical scenario. These models mimic stimuli hypothesised to be present during infection and may be divided into two groups: (1) Those designed to generate a largely homogenous bacterial population to characterise specific responses and develop drug screens for defined bacterial phenotypic states. (2) Those aimed at producing mixed populations of *M.tb*, often with multiple stimuli to model drug action on heterogeneous populations. Of course, bacterial heterogeneity is entirely defined by the methods used to characterise *M.tb* populations and the resolution of the techniques.

The method described by Wayne and colleagues is the most frequently used experimental approach to hypoxia and, hence, the best characterised. In this model, *M.tb* is grown under agitation in air-tight containers with a defined headspace-to-culture ratio and for a defined period of time (usually 24 days), which leads to the gradual depletion of oxygen.[23] When deprived of oxygen, the bacilli enter a non-replicative state that is refractory to isoniazid-dependent killing.[36] This physiological state may be reversed by exposure to atmospheric oxygen conditions.[22,37] The Wayne model has been used for the evaluation of new compounds; non-replicating phase 2 (NRP2) bacilli were treated with test drugs and subsequent mycobacterial growth was determined by conventional plating methods.[38] Using similar methodology, metronidazole and PA-824 (a nitroimidazo-oxazine now in phase 2 and 3 clinical trials) were shown to be active against anaerobic *M.tb in vitro*.[38] Several versions of the Wayne model have been developed to increase its throughput capacity, for example, by combining with colorimetric or luminescence-based measures of bacterial viability.[35,39,40] In addition, using multiple genome-wide analyses, Galagan and colleagues have been able to explore the molecular mechanisms that are employed by *M.tb* during hypoxia and reaeration phases.[41]

Deprivation of nutrients is another stress hypothesised to be encountered by *M.tb* inside granulomas. The models that reproduce this condition normally involve the incubation of tubercle bacilli in minimal medium for approximately 6 weeks. During this period, the cells undergo a global metabolic shift:[42,43] several metabolic pathways are shut down and lipids become the sole source of energy;[44] while rescue pathways, such as those involved in the synthesis of vital enzymes, are upregulated. Aerobic respiration usually shuts down after ~9-12 days. Starvation-induced persistent-bacilli are tolerant to some antimicrobials, such as isoniazid, rifampicin and metronidazole. However, pyrazinamide, econazole and clotrimazole are active against this *M.tb* population.[42,45,46] Chemostat models are a key resource for such investigations where a controlled environment is achieved by fine-tuning bacterial growth rate alongside parameters affecting the respiratory and metabolic state of bacilli. Chemostats have been successfully used to study the molecular adaptations of mycobacteria to nutrient depletion,[47] low oxygen tension,[48] and between fast-growing and slow-growing populations.[49] Additional single stress models have been developed by either limiting the availability of specific nutrients or inducing a stress predicted to be present *in vivo*, for example low potassium,[50] PBS starvation model,[42] reduced oxygen, low pH.[51,52]

Hypoxia and nutrient starvation models can trigger *M.tb* to slow growth and activate the DosR/DevR regulon, however they cannot simulate the multiple environmental stimuli that are likely found within granulomas, as bacilli adapt to the dynamic surroundings. For this reason, multiple-stress models (combining hypoxia, nutrient starvation, low pH) may offer a more complete *in vitro* simulation of the circumstances bacilli encounter in some human lung lesions.[53] The model developed by Deb *et al.* was shown to