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Association of pellicle growth morphological characteristics and clinical presentation of *Mycobacterium tuberculosis* isolates

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S U M M A R Y

Trehalose 6,6'dimycolate (TDM) is a glycolipid found in nearly pure form on the surface of virulent *Mycobacterium tuberculosis* (MTB). This manuscript investigated the production of TDM, growth rate and colony morphology of multiple strains of MTB, each of which had been isolated from both pulmonary (sputum) and extrapulmonary sites of multiple patients. Since sputum contains MTB primarily from cavities and extrapulmonary biopsies are typically granulomas, this provided an opportunity to compare the behavior of single strains of MTB that had been isolated from cavities and granulomas. The results demonstrated that MTB isolated from pulmonary sites produced more TDM (3.23 ± 1.75 μg TDM/mg MTB), grew more rapidly as thin spreading pellicles, demonstrated early cording, and climbed culture well walls. In contrast, extrapulmonary isolates produced less TDM (1.42 ± 0.58 μg TDM/mg MTB) ($p < 0.001$) and grew as discrete patches with little tendency to spread or climb. Both Beijing pulmonary isolates and the non-Beijing pulmonary isolates produced significantly more TDM (1.64 ± 0.46 μg TDM/mg MTB) and grew faster than the Beijing and non-Beijing extrapulmonary isolates (1.14 ± 0.63 μg TDM/mg MTB) ($p < 0.001$ and $p < 0.005$ respectively). These results indicate that MTB from pulmonary sites (cavities) grows faster and produces more TDM than strains isolated from extrapulmonary sites (granulomas). This report suggests a critical role for TDM in cavitary TB.

1. Introduction

Tuberculosis (TB) remains an urgent global health crisis, with nearly a third of the world's population infected with the causative pathogen, *Mycobacterium tuberculosis* (MTB). In 2013, there were 9 million cases of TB disease and 1.5 million deaths (http://www.cdc.gov/tb/statistics/default.htm). One of the major obstacles to developing more effective vaccines and/or therapeutics is the continued lack of understanding of the pathogenesis of TB disease. It is now widely accepted that MTB infection promotes diverse microenvironments in the same host. However, MTB can only spread from patient to patient when a cavity forms in the lungs. Thus, understanding the mechanism of how MTB induce cavity formation is critical towards eliminating TB disease.

By studying lung samples collected from individuals whom have died from TB disease during autopsy, it has become clear that cavity formation most likely develops from necrosis of obstructive lobular pneumonia, which results from accumulation of foamy alveolar macrophages in alveoli and the bronchi [1]. The process of foamy alveolar macrophage formation is hypothesized as a crucial point in the pathology of cavity formation and TB disease transmission. MTB is known to hijack the host macrophage lipid metabolism machinery, allowing accumulation of host lipids as a mechanism to enter into dormancy and/or to store necessary nutrients for its survival [2–6]. However, published and recent evidence suggest that foamy alveolar macrophage also accumulates lipids and antigens from MTB [7–9].

One such MTB component that may be important in the promotion of cavitary disease is Trehalose 6,6′ dimycolate (TDM), or cord factor. TDM is arguably the oldest and most studied virulence factor of MTB. Koch reported that MTB aggregate in rope like structures known as serpentine cords [10,11]. In 1947, Middlebrook, Dubos and Pierce reported that the ability of MTB to form such

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cords is an 'essential accompaniment of virulence' [12]. Following suggestions that cord formation was due to a lipid substance on the surface of the organism, Block isolated and later characterized a lipid that he named cord factor. It was later identified as TDM [13,14]. TDM is the only lipid detectable by TLC that can be extracted from the surface of virulent MTB without killing the organisms [15–21]. It can also cause beads to aggregate into structures resembling cords [16]. TDM is critical for MTB survival, preventing phagosome-lysosome fusion events and activating intracellular killing mechanisms. Removing TDM from the surface of MTB reduces the ability to survive in macrophage culture and in mice [15,19,20]. The evidence that TDM plays important roles in infection with MTB is extensive and compelling and is reviewed elsewhere [22].

The presence and amount of TDM can influence the morphology of virulent isolates of MTB [18]. MTB constantly produces TDM, and the excess is released from the surface into the surrounding environment. Preliminary laboratory observations (Ranjana Arora, personal observation) showed that the amount of TDM produced influences colony morphology. When grown as a submerge culture, MTB routinely releases TDM into the liquid medium. This phenomenon is hypothesized to occur during MTB infection in lung tissue, and that this release and buildup of TDM antigen in the tissue microenvironment may lead to cavity formation, a necessity for disease transmission [22,23].

In the human lung, the released TDM is hypothesized to be responsible for the development of disease pathogenesis by inducing various immune responses, including memory immunity. Purified TDM coated on beads or emulsified in oil/water vehicle induces granulomatous pathology in lung tissue of mice [24–27]. Additionally, immunization with TDM can generate a transferrable memory $CD4^+$ T cell response [28,29], and this TDM hypersensitive response is species specific. Challenge with TDM from attenuated *Mycobacterium bovis* Calmette Guerin Bacillus (BCG) in mice immunized with TDM from MTB does not generate the TDM hypersensitive response [30]. This evidence suggests that in the human lung, MTB favors production of large quantities of TDM to facilitate its survival and eventual transmission. The role of TDM could be the key to understanding tubercular pathogenesis.

TB disease can have varied clinical presentation in terms of severity as well as location (pulmonary infection or infection of extrapulmonary sites) [24]. This suggests that MTB residing in lung tissue has adapted specific responses to the localized environment to facilitate transmission, which is believed to be facilitated by TDM. This study investigates 90 de-identified clinical isolates of MTB representing 12 strains, each of which was isolated from multiple sites from different people. TDM was isolated from MTB belonging to the same spoligotype collected from different sites of the patient (pulmonary versus extrapulmonary), and assessed according to type of organism (Beijing or non-Beijing). The clinical isolates were grown as pellicle cultures that were analyzed for the timing and characteristics of pellicle growth in addition to production of TDM.

2. Materials and methods

2.1. Study design

The study was a longitudinal laboratory study that evaluated association between TDM produced by MTB strains collected at pulmonary or extrapulmonary sites. The sampling frame included a list of de-identified clinical isolates of *Mycobacterium tuberculosis* (MTB) along with details of site of isolation and virulence genetic group which was based on spoligotyping. The isolates were chosen from genetic groups to represent Beijing and Non-Beijing families [31].

The clinical isolates were grown as pellicle cultures in an effort to get the most accurate total of TDM produced. As TDM is constantly released from the MTB cell surface, submerged cultures with surfactant releases TDM into the culture broth, where it would be more difficult to isolate. As a pellicle culture, the released TDM spreads as a thin film on the air-water interphase of the culture broth, allowing collection of TDM that is no longer associated with the MTB cell wall. Clinical isolates that were not able to be grown as a pellicle culture were eliminated from further evaluation.

2.2. Culture

Middlebrook 7H9 broth (Remel, PA) with 10% supplement containing 5% bovine serum albumin (BSA, Sigma) and 2% dextrose (Sigma) was used for surface pellicle growth. No Tween 20 was added. A pellet of organisms was suspended in 1 ml of sterile water, sonicated, and centrifuged at 13000 rpm for 5 min. The organisms were then re-suspended in 200 μl petroleum ether. The re-suspended sample was divided into two equal parts to initiate the cultures in duplicate in 6-well plates with 6–8 ml of Middlebrook 7H9 broth in each well. Cultures were grown at 37 °C with 5% CO_2.

2.3. Pellicle morphology

The cultures were observed microscopically at 20× magnification, as well as macroscopically, twice a week for six weeks. Growth characteristics observed included duration for appearance of growth (in weeks), cording (early or late), spreading (continuous or non-continuous), climbing (presence or absence), and thickness (thin or thick). These characteristics were noted on a spreadsheet for all isolates for statistical analysis. Duration of appearance of growth was recorded as time (in weeks) when growth became visible under a phase contrast microscope at 20× magnification. This was recorded as 1 week, 2 weeks, 3 weeks, and 4 weeks, following which the strain were entered as no growth. Early cording was described as appearance of cording on 20× magnification under a phase contrast microscope in less than 1 week of appearance of growth. Continuous growth was described as a non-discreet film of growth on the surface visible macroscopically, while climbing growth was depicted by macroscopically visible growth on the walls of the well.

2.4. TDM extraction

Four week old cultures were harvested into pre-weighed glass tubes, centrifuged to a pellet, washed and centrifuged again at 2500 rpm for 30 min to remove the media. Surface TDM was extracted 3 times using petroleum ether and carried out as previously described[12]. The extract was then solubilized in chloroform-methanol (2:1) and then analyzed by thin layer chromatography (TLC).

2.5. Thin layer chromatography (TLC)

TLC was standardized and compared against a TDM standard (Sigma). TLC was performed on Whatman K6F silica gel, 5 × 10 cm, 60 A plates. Each plate was inoculated with 10 μl each of a standard TDM (diluted to 1 mg/ml) and 2 clinical isolates. The plates were run in elution buffer: chloroform, methanol, and ammonium hydroxide (90:10:1). TLC visualized with 5% sulfuric acid- 0.5% alpha napthol in 100% ethanol as the charring reagent. Amount of TDM was normalized against the wet weight of the MTB pellet taken prior to TDM extraction. Serial dilutions of the TDM extracted from