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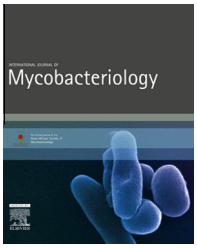

Nontuberculous mycobacteria pathogenesis and biofilm assembly

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ABSTRACT

Nontuberculous mycobacteria (NTM) are emergent pathogens whose importance in human health has been gaining relevance after being recognized as etiological agents of opportunist infections in HIV patients. Currently, NTM are recognized as etiological agents of several respiratory and extra-respiratory infections of immune-competent individuals. The environmental nature of NTM together with the ability to assemble biofilms on different surfaces plays a key role on their pathogenesis.

In the present work the ability of three fast-growing NTM (*Mycobacterium smegmatis*, *Mycobacterium fortuitum* and *Mycobacterium chelonae*) to persist within a model of human alveolar macrophages was evaluated. Most often human infections with NTM occur by contact with the environment. Biofilms can work as environmental reservoirs. For this reason, it was decided to evaluate the ability of NTM to assemble biofilms on different surfaces. Scanning electron microscopy was used to elucidate the biofilm structure. The ability to assemble biofilms was connected with the ability to spread on solid media known as sliding. Biofilm assembly and intracellular persistence seems to be ruled by different mechanisms.

Introduction

Nontuberculous mycobacteria (NTM) are a heterogeneous group composed of 169 different species of environmental organisms commonly isolated from water, soil, dust and diverse animals (http://www.bacterio.net/mycobacterium.html). For this reason, their isolation in clinical samples was regarded as contamination or colonization for a long time [1,2]. Human infection with NTM became relevant with the emergence of the human acquired immune deficiency syndrome pandemic. Mycobacteria from *Mycobacterium avium* complex (MAC) were identified as the major cause of opportunistic infections in patients infected with the human immunodeficiency virus (HIV). In contrast to *Mycobacterium*

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tuberculosis, reporting of NTM infections is not mandatory; thus, precise incidence data are lacking. Despite this fact, NTM are recognized as etiological agents of healthcare-associated infections (HAIs), which are a major public health concern [3,4]. These bacteria are often responsible for respiratory tract colonization/infection; infections related to medical procedures and disseminated infections in immunocompromised patients. Although *M. avium* remains the best-known NTM, rapidly growing bacteria, namely *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium abscessus*, are gaining growing relevance [5–7].

Biofilm formation is a successful survival strategy for these ubiquitous organisms found in the environment. Biofilm assembly proceeds through several phases, including reversible attachment, irreversible attachment, mature biofilm formation and dispersion. During this process, bacteria develop a matrix containing extracellular polymeric substances, such as polysaccharides, lipids and nucleic acids, to form a complex three-dimensional structure.

NTM, organized in biofilms, are difficult to eradicate with common decontamination practices and are relatively resistant to standard disinfectants [8,9], such as chlorine, organomercurials, and alkaline glutaraldehydes [8,10–12]. It is important to eradicate biofilms through agents that inhibit biofilm formation, using two main strategies: matrix synthesis and regulatory mechanisms [9]. Biofilms can be also very resistant to high concentrations of antimicrobial drugs and are able to modulate the host immune system [13]. This high resistance is mainly due to the virulence enhancing caused by biofilms. Bacteria within biofilm are more prone to horizontally exchange genes due to the maximum proximity between them [14]. This gene transmission is a major cause for bacteria survival [15] and can account for a high frequency of mutations responsible for antimicrobial resistance [16]. These mutations can trigger enzymatic production, modification of antibiotic target or formation of efflux pumps [17–19]. Bacteria can also switch their phenotypic stages causing a slower growth rate. This strategy will decrease, or even inhibit, the antibiotic efficiency of agents active on replicating microorganisms. The bacteria that adopt this behavior are called "persisters" [20]. The depletion of nutrients in the center of the biofilm can also affect bacteria growth, having an impact on persisters [21].

Bacteria cell-to-cell communication, known as quorum-sensing, involves the production of auto-inducer molecules (AIs) which mediate the behavior of the bacterial population [9]. Many biological processes, e.g., biofilm formation or the expression of virulence factors, are controlled by quorum-sensing regulation [9]. The self-produced matrix is also considered important in enhancing bacteria virulence. The matrix builds a barrier that can inactivate antibiotics, delaying or preventing antibiotic penetration within the biofilm and recognition of their targets [22].

Many human diseases are caused or exacerbated by biofilms [9], and it is expected that as in other infectious diseases (e.g., urinary infections), biofilms provide an important reservoir for cells that can repopulate colonized sites upon removal of drug treatment [23,24]. Nevertheless, the mechanism involved in biofilm assembly and persistence are far from being fully elucidated.

Research on NTM biofilm is still in its infancy. The majority of the published studies were conducted on the model organism *Mycobacterium smegmatis*. It was shown that the genetic requirements for sliding motility on agar surfaces and biofilm formation are similar [25]. The presence of specific cell wall components, such as glycopeptidolipids (GPL) [26], other factors such as GroEL1 [27], protein kinase [28], iron [29] or the lack of others, e.g., polyphosphate deficiency [30], affect biofilm formation, matrix composition and structure. The role played by cell wall GPL was also reported for *Mycobacterium avium* [31] and *M. abscessus* [32].

Most of the studies in the biofilm field in general were focused on the identification of factors involved in the first phase of biofilm assembly (attachment to a surface). Protein adhesions required for irreversible surface adhesion have been identified, but there is little direct experimental support for these surface proteins mediating cell–surface interactions. The aims of this study are: to investigate the relationship between NTM biofilm assembly and intracellular persistence; and to characterize the ultra-structure of the biofilms assembled by the different NTMs enrolled in the present study.

Materials and methods

Bacterial culture conditions

M. smegmatis mc^2 155, *M. fortuitum* ATCC 6841 and *M. chelonae* ATCC 35752 were grown on Middlebrook 7H9 broth medium (Difco, Becton, Dickinson & Company, Sparks, MD, USA) supplemented with 10% OADC (v/v; Difco) and 0.05% Tween 80 (v/v; Sigma, St. Louis, MO, USA) until the exponential phase at 37 °C/5% CO_2. At this point, single cell suspensions were prepared as described before [33] and stored at −80 °C in 50% glycerol (v/v; Sigma) solution in phosphate buffer saline (PBS) until further use.

Cell line culture conditions

THP1 cells (ATCC TIB-202) were grown in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS; Lonza), 2 mM L-glutamine (Gibco, Life Technologies Corporation, Grand Island, NY, USA), 10 mM Hepes (Gibco), 1 mM sodium pyruvate (Gibco), 4500 g/mL glucose (Gibco), and 50 μg/mL gentamicin (Gibco) at 37 °C/5% CO_2. The cells were seeded onto 96-well culture dishes at a density of 4×10^4 cells per well and treated for 72 h with 100 nM phorbol myristate acetate (Sigma). Then cells were washed three times with PBS and incubated for one more day in cell culture media without phorbol myristate acetate.

Macrophage infection

Mycobacteria stocks were thawed and diluted in RPMI 1640 without gentamicin to an optical density (OD) at 600 nm of 0.1. Macrophages were then infected as described before [33]. At infection time (1 h) and after several hours (4, 8, 24 h), infected macrophages were washed with PBS and lysed with an aqueous solution of 1% igepal (v/v; Sigma). Serial dilutions of the lysate were prepared in water and plated on Muel-