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Looking in amoebae as a source of mycobacteria

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ABSTRACT

Mycobacteria exhibit various relationships with amoebae, ranging from the killing of one partner by the other one, to amoebae hosting mycobacteria in trophozoites and cysts. This observation indicates that poorly described biological factors affect the relationships, including mycobacterial cell-wall glycolipids and the size of the mycobacteria. Experimental observations indicate that a majority of environmental, opportunistic mycobacteria but also obligate pathogens including *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans* are inter-amoebal organisms. Amoebae may give opportunities for genetic exchanges between mycobacteria, sympatric intra-amoebal organisms and the amoebae themselves. Amoebae clearly protect opportunistic mycobacterial pathogens during their environmental life but their role for obligate mycobacterial infection remains to be established. Accordingly, water was the source for emerging, community-acquired and health care-associated infection with amoeba-resisting mycobacteria of the *Mycobacterium avium, Mycobacterium abscessus* and *Mycobacterium fortuitum* groups, among others. Amoebae are organisms where mycobacteria can be found and, accordingly, amoeba co-culture can be used for the isolation of mycobacteria from environmental and clinical specimens. Looking in amoebae may help recovering new species of mycobacteria.

1. Introduction

Mycobacteria are actinomyces organisms of medical and veterinary interest, responsible for opportunistic and obligate infections in birds and mammals [1]. In humans, obligate infections comprise severe and deadly tuberculosis due to members of the *Mycobacterium tuberculosis* complex, leprae due to *Mycobacterium leprae* and Buruli ulcer due to *Mycobacterium ulcerans*. The most recent common ancestor of modern-day mycobacteria was unlikely a host-adapted organism, but rather an environmental organism [2,3] which evolved towards soil-borne mycobacteria such as *Mycobacterium intracellulare*, waterborne mycobacteria such as *Mycobacterium avium* and host-associated species including *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* [4], *M. tuberculosis* complex species and *M. leprae*. Some mycobacteria retained genes for dual lifestyle as in the case of *Mycobacterium marinum* [3]. In the latter species, genome organization and size are more related to the saprophyte *Mycobacterium smegmatis* than to *M. tuberculosis*, despite the fact that *M. marinum* and *M. tuberculosis* share 80% of coding sequences [3]. Also, transfer of the IS6110 insertion sequence between *M. smegmatis* and the *M. tuberculosis* complex species and its discovery in the environmental *Mycobacterium* sp. strain JLS, suggest that ancestors of these mycobacteria once lived into the same environmental niche [5]. Recent analysis of mycobacteriophages in the *Mycobacterium abscessus* complex reinforces this view [6]. Indeed, even the highly specialized *M. tuberculosis* complex mycobacteria [7] and *M. leprae* [8] retain some capability to survive in soil.

Of 140 species currently described in the genus *Mycobacterium*, 133 (95%) species are environmental organisms [9–11] whereas seven are host-associated organisms. In the environment, the common ancestor and derived mycobacteria were likely in contact with unicellular eucaryotes including amoebae and contact with amoebae likely also contributed to shape the evolution of mycobacteria [12].

1.1. Environment, amoebae and mycobacteria

Amoebae are cell-wall-free, unicellular eukaryotes that can switch from a motile trophozoite form towards an immobile cyst during starvation, drying, hypoxia and fluctuation in temperature. Trophozoites are professional phagocytes that engulf any particle with a diameter ≥0.5 μm into phagocytic vacuoles with further

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lysosome fusion and destruction. Encystement consists of the formation of a cell wall comprising one layer as in the case of *Entamoeba* amoeba and two layers as in the case of *Acanthamoeba* amoeba and *Hartmannella vermiformis* [13]. In *Acanthamoeba*, the cell wall is composed of cellulose, a polysaccharide with β-configuration [14]. Cysts are resistant to alterations in osmolarity and pH, desiccation, freezing, high concentration of hydrochloric acid, moist heat, chemical antimicrobial agents and biocides [15–17]. Accordingly, cysts can survive for several decades [18]. Excystment refers to the process by which trophozoites emerge through ostioles of the amoebal cyst, a processus requiring the action of cellulases.

Although mycobacteria have been rarely and convincingly detected inside amoebae collected in the field, there are few direct evidences of mycobacteria in naturally-infected amoebae. That was the case of poorly identified mycobacteria more closely related to *Mycobacterium intracellulare*, which was clearly observed within and cultured from *Acanthamoeba lugdunensis* recovered from a contact lens solution [19]. Further indirect data came from the repeated observation of mycobacteria in the same environment where amoebae have also been recovered [20,21]. These environments include soil and fresh water [22], cooling towers [23], municipal potable water systems [24,25] and hospital water distribution systems [26,27]. Accordingly, 13 different *Mycobacterium* species have been isolated from the very same specimens where *Hartmannella*, *Echinamoeba*, *Acanthamoeba* and *Naegleria* amoeba have also been isolated [25]. Also, the detection of *H. vermiformis* and *Acanthamoeba polyphaga* significantly correlated with the detection of mycobacteria (*Mycobacterium gordonae*, *Mycobacterium xenopi* and *Mycobacterium kansasii* subtype 1) in a hospital water network [26]. Likewise, several mycobacteria but not *M. ulcerans*, have been isolated from protected and unprotected water sources in Benin where *Acanthamoeba*, *Naegleria* and *Tetramitus* amoeba have also been detected [24] (Table 1).

1.2. Mycobacteria–amoeba interactions

Laboratory experiments confirmed field observations that some mycobacteria are amoeba-resisting organisms [28,29], meaning that mycobacteria have evolved mechanisms to resist these unicellular protist predators, including the capacity to avoid lysosomal killing and to replicate intracellularly within protozoa. Mycobacteria gained the advantage of being protected from adverse conditions by the amoebal cyst which is acting as a "Trojan horse" protecting mycobacteria. Laboratory investigations used waterborne amoebae, including *Acanthamoeba polyphaga* [30–32], *Acanthamoeba castellanii* [33–37], *Hartamnella vermiformis* [28,29,38], and the social soil amoeba *Dictyostelium discodium* [39,40]. The ciliated protozoan *Tetrahymena pyriformis* has also been used to study the intracellular growth of the *M. avium* complex species [40]. In particular, *Acanthamoeba* amoeba develop at 32–37 °C, a temperature range for natural mycobacteria–macrophage interactions [34]. Different multiplicities of infection (MOI) from 1:100 [31] to 1:10 [42,43] are most often used with removal of extra-amoebal mycobacteria using buffer [31], or antibiotic (mainly an aminoglycoside) [33,29] or acidity [31]. The growth of mycobacteria can be measured inside the trophozoite by microscopic counting of intra-amoebal mycobacteria after Ziehl [43] or fluorescent staining [41], electron microscopy counting [30], counting mycobacteria colonies released from the amoeba, or quantitative real-time PCR.

Membrane interactions and the resulting engulfment of mycobacteria into the amoebae [44] as well as subsequent mechanisms for bactericidal effect [45] have been studied. Co-culture of mycobacteria with these amoebae may result in either amoebae killing by the mycobacteria, mycobacteria killing by the amoebae or any sympatric living between the organisms in the case of the so-called amoeba-resisting mycobacteria (Fig. 1).

The composition in cell wall lipopolysaccharides influences the phagocytosis of mycobacteria. This is illustrated by the observation that *Mycobacterium bovis* is phagocyted by *Acanthamoeba castellanii* whereas the BCG clone, derived from *M. bovis* by genome reduction, is not phagocyted in the same model [46] (Table 1). This observation therefore suggests that some genetic determinants may drive the phagocytosis of mycobacteria by amoebae. It has been observed that mycobacterial cell wall composition acts as antiphagocytosis factors in co-culture of *M. marinum* by amoebae including *Dictyostelium discoideum*, *Acanthamoeba catsellanii* and *A. polyphaga* [47,48]. These data correlate with observations in macrophages [48] and the zebrafish [47], indicating that amoebae are an appropriate model organism to screen mycobacteria for host interactions. However, we observed the opposite in the *M. tuberculosis* complex; indeed, we observed that the smooth-colony variant *Mycobacterium canettii* was significantly more readily phagocytosed by *A. castellanii* than the rough-colony variants *M. tuberculosis* and *M. bovis*; the percentage of phagocytosed inoculum was respectively 89/0.6%, 12.4/0.3% and 11.7/0.2% [49].

Killing of amoeba results from the extensive multiplication of intracellular mycobacteria and indeed, the amoeba itself may promote the growth of its invader [50]. Accordingly, the mycolactone toxin synthesized by a plasmid-encoded polyketide synthase of *M. ulcerans* [51] is not cytotoxic against *D. discoideum* [52]. Interestingly, we observed that mycobacteria <2 μm do not multiply within or kill the amoebae; whereas the opposite is observed for mycobacteria >2 μm [53]. Once inside the amoebae, mycobacteria reside in vacuoles (Fig. 2).

As for mycobacteria residing for longer periods into amoebae, we observed that amoebae were a place for genetic exchanges between the mycobacteria, other symbiotic intraamoebal organisms and the amoeba itself [12]. In this study, the phylogenetic analysis of the gene encoding a pyridine nucleotide disulfide oxidoreductase (pyr-redox) yielded a non-conventional topology, placing non-tuberculous mycobacteria in a clade with the intra-amoebal gamma-Proteobacteria Legionella, the ciliate *Tetrahymena thermophila* and *D. discoideum*. Furthermore, co-culture of *M. avium* and *Legionella pneumophila* within *A. polyphaga* confirmed the biological relevance of this observation [12]. Further, intraamoebal location has been shown to increase the virulence of *M. avium*, in terms of enhanced entry into cells and in a mice model [33].

Some mycobacteria are encysted with the host (Table 1). We showed that compared to other bacteria, which are residing within the cytoplasm, *M. avium* complex mycobacteria are residing in the wall of the cysts [54]. We observed that, unexpectedly, mycobacteria of the *M. tuberculosis* complex do encode for a set of 3 functionnal cellulases which are enzymes mainly devoted to the digestion of plants [55–56].

Mycobacterial cellulases may play a role in the encystment and de-encystment of the amoebae indured by mycobacteria.

1.3. Amoebae and community-acquired mycobacterial infections

In the community, the first cases of *Mycobacterium fortuitum* furunculosis were reported after attendance of nail salon in the United States [57]. A large outbreak investigated in California was due to contamination of the water footbath with *M. fortuitum* and razor shaving was a risk factor for lesions [57]. *Mycobacterium mageritense* was responsible for another, similar situation in another nail salon [58]. Both organisms have been shown to be amoeba-resisting mycobacteria (Table 1). Also, contact lens storage cases were shown to contain *Acanthamoeba* amoeba, and

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