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Survival of pathogenic *M. abscessus* subsp. *massiliense* in *Acanthamoeba castellanii*

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Abstract

We used an amoeba model to study the intracellular growth and cytotoxicity of clinical strains of *M. abscessus* subsp. *massiliense* (Mabsm) isolated from 2 patients (one with cystic fibrosis, the other one with idiopathic bronchiectasis) during the early (smooth colonies) and late stage (rough colonies) of chronic pulmonary infection. *Acanthamoeba castellanii* were infected with Mabsm (MOI 100) and samples collected every 24h for 72 h. Results showed Mabsm is able to survive in trophozoites and persist in cysts for at least 7 days. Late Mabsm demonstrated higher cytotoxicity toward *A. castellanii* when compared to early strains. *A. castellanii* is a useful *in vitro* host model to study infection of Mabsm clinical isolates.

Keywords

Acanthamoeba castellanii; *Mycobacterium abscessus*; chronic lung infection

1. Introduction

Mycobacterium abscessus complex (*M. abscessus* subs. *abscessus*, *M. abscessus* subs. *massiliense*, *M. abscessus* subs. *bolletii*) is an important rapidly-growing mycobacteria group implicated in chronic infections of skin, soft tissues, and lungs [1, 2]. The prevalence of *M. abscessus* has steadily increased in last decades in immunocompromised persons and those underlying structural lung diseases such as cystic fibrosis [3].

M. abscessus can display a rough or smooth colony morphology. The smooth phenotype expresses glycopeptidolipid (GPL) in the cell wall envelope which confers sliding motility and biofilm formation [4]. Their ability to colonize surfaces is an important feature to thrive

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in the environment [5]. Smooth *M. abscessus* is frequently found in wound infections and in early stages of lung infection [5].

In patients with chronic lung infections with *M. abscessus* increase presence of rough colony morphotype in culture seems to be associated with disease progression [5]. *M. abscessus* with a rough morphotype do not present sliding motility and are unable to form biofilm. However, this morphotype induces higher inflammatory responses than the smooth morphotype, and it presents an invasive behavior with cording formation associated with disease persistence in vivo [6, 7].

The interactions between nontuberculous mycobacteria (NTM) and amoebae have gained importance in recent years due to their co-occurrence in nature, suggesting that amoebae may serve as a reservoir of mycobacteria [8, 9]. Amoebae have additionally being recognized as an important host for mycobacteria to develop mechanisms of virulence and resistance replicated within human macrophages [8].

The purpose of this study was to evaluate the infectivity, cell association, and intracellular growth rate of *M. abscessus* subsp. *massiliense* (Mabsm) strains isolated at early and late stages of lung infection from 2 patients with different underlying disease (cystic fibrosis or idiopathic bronchiectasis) with worsening clinical status.

2. Methods

2.1 Mycobacteria

The Mabsm clinical strains were provided by the Microbiology Service, Department of Laboratory Medicine, National Institutes of Health, USA. A total of 4 strains were isolated at early and late stages of infection from two patients with worsening clinical status. Mabsm B1 (smooth colony) was isolated from a patient with cystic fibrosis in June 2000. This isolate evolved to a rough morphotype, resistant to clarithromycin and amikacin (Mabsm B8). Strain B8 was implicated in the lung infection outbreak in a cystic fibrosis center in Seattle, Washington, USA [10, 11]. The patient underwent lung transplantation, but died due to disseminated infection. Mabsm RS1 (smooth colony) was isolated in April 1998 from a patient with bronchiectasis. This strain also evolved to a rough morphotype (RS8), isolated in August 2009, resistant to clarithromycin, but not to amikacin.

Strains were grown at 37° C, 150rpm, until they reached an Optical density (O.D) of 0.8 in 7H9 supplemented with 10% ADC (Sigma Aldrich, St Louis, Missouri, EUA), and 0.05% tyloxapol (Sigma Aldrich). Mycobacteria were washed and suspended in 20 mL of Phosphate Buffer Saline (PBS) pH 7.2 with 0.05% Tyloxapol, sonicated for 30 sec, with 10 sec intervals and used to infect *A. castellanii*.

2.2 *Acanthamoeba castellanii*

Acanthamoeba castellanii ATCC 30010 were grown in 10 mL of Peptone-Yeast extract-Glucose broth (PYG) [14] in 25 cm² culture flasks at 28°C. Confluent cells were harvested, centrifuged at 500 g for 10 min and suspended in fresh PYG medium with 10% DMSO (v/v) to obtain a final concentration of 10⁷ amoebae cells/mL. Stock solutions were kept in

–80 °C for 3 h and transferred to liquid nitrogen. For experiment, aliquots were thawed at 37°C and grown as previously described. Amoebae were discarded after every three experiments.

2.3 Infection of *Acanthamoeba castellanii*

A. castellanii trophozoites grown in Peptone-Yeast Extract-Glucose (PYG) were transferred to 24-well plates at $\sim 10^6$ cells/mL and incubated at 28.5°C overnight. Amoebae cells were washed three times with Page's modified Neff's amoeba saline (PAS) and infected with mycobacteria (MOI 100). After 20-min centrifugation at 500 rpm, infection was maintained for an additional hour. Plates were gently washed three times with PAS, PAS removed and 1 mL of fresh PYG media was added.

2.4 Proportion of amoebae associated with mycobacteria

PYG media was removed from infected amoeba cells (after 1h infection = Time 0) and 500 μ L PBS were added. Cells were mechanically detached from the plate (volume 500 μ L PBS) and 100 μ L were transferred to microscope slides for staining. Cells were stained with Remel TB Kinyoun AFB Stain Kit (ThermoFisher, Waltham, Massachusetts, EUA). The percentage of infected amoebae was determined by counting two hundred cells using light microscopy and defined as the number of infected amoebae divided by the total number of amoebae counted multiplied by 100 [12, 13].

2.5 Mycobacteria growth in infected amoeba

Following infection (Times 0, 24, 48, 72 h), PYG media was removed and adherent *A. castellanii* gently washed three times with PBS. Attached cells were incubated for 10 min with 500 μ L of cold 5 mM EDTA [14], transferred to 1.5-ml Eppendorf tubes, and washed three times (20 min 1000 g) in PBS. Pelleted *A. castellanii* were lysed by addition of 500 μ L 0.1% (v/v) SDS in water for 30 min and passed through a 26-gauge needle [6] to obtain resuspended bacteria. Ten-fold dilutions were performed and 100 μ L mycobacteria plated in 7H11 agar plates (ThermoFisher) and colonies counted after 5 days incubation at 37°C. Results were expressed in CFU/mL.

2.6 Lactate Dehydrogenase release assay

The LDH release assay was performed on PYG supernatants after 72h infection. [8] using CytoTox 96 NonRadioactive Cytotoxicity Kit (Promega, Madison, WI) following manufacturer instructions.

2.7 Survival of mycobacteria in encysted *Acanthamoeba castellanii*

A. castellanii cells were infected with mycobacteria as described above and incubated for 7 days. Cysts were extensively washed with PAS and treated with 100 μ g hygromycin for 1 h in order to kill extracellular mycobacteria and plated onto 7H11 to assess mycobacterial growth.

2.8 Statistical analysis

Results are shown as the mean $\pm$ standard deviation of three experiments performed in different days. Significance (p-value < 0.05) was determined by paired Student's t-test.

3. Results and Discussion

Previous studies have provided insights on survival of mycobacteria in amoebae [8, 9] but just a few have studied interaction of pathogenic *M. abscessus* with *A. castellanii* [15]. This is the first study evaluating survival of *M. abscessus* subsp. *massiliense* isolated from patients in different time points of their disease course.

The proportion of amoebae associated with mycobacteria was similar for Mabsm smooth variant B1 (69 %) and rough variant B8 (60%) (p > 0.05). Similar data were observed for Mabsm RS1 (smooth) and RS8 (rough), 67% and 62 % (p > 0.05), respectively. These data suggest that attachment to *A. castellanii* is not significantly impaired by the rough characteristic of the mycobacteria.

Mabsm B1 had a faster growth rate in *A. castellanii* when compared to Mabsm B8, reaching almost a ~ 1 log difference after 72 h infection. A similar difference was observed between RS1 and RS8 (Fig. 1). While both Mabsm B8 and RS8 strains showed slow growth and rough colony morphology, B8 was much rougher and formed clumps (Fig 2), a characteristic correlated with higher pathogenicity in macrophages [16] and zebrafish [6].

As previously described for *M. avium* complex [17], we observed Mabsm entrapped in 7-day old encysted amoebae. In order to determine if trapped mycobacteria remained viable, cysts were pretreated with hygromycin and plated onto 7H11 for up to 15 days at 37 °C. Growth of Mabsm was observed for all 4 strains tested consistent with survival of mycobacteria upon both *A. castellanii* ingestion and encystation. Such survival characteristics may protect *M. abscessus* subsp. *massiliense* against harsh environmental conditions as also observed with other mycobacteria [18] and could facilitate transmission. Environmental sources are presumably the source of *M. abscessus* skin and soft tissues infections [3] which have been reported following invasive medical procedures. While lung infections have been thought to be acquired from environmental sources, recent reports raised the possibility of patient-to-patient transmission [11, 19].

Cytotoxicity to amoeba as measured by LDH release was higher in *A. castellanii* infected with rough strains Mabsm B8 and Mabsm RS8 than smooth strains Mabsm B1 and Mabsm RS1, respectively, consistent with a higher virulence of strains with the rough morphotype (Fig. 3). Consistent with our findings, *M. abscessus* rough morphotypes have been shown to form large clumps within the phagocytic vesicles ultimately killing J774 macrophages [16]. In zebrafish models, rough *M. abscessus* can escape macrophage engulfment by formation of large cords, allowing its spread to other tissues [6].

M. abscessus requires prolonged and often indefinite drug treatment; failure to eradicate the infection may lead to genetic changes in mycobacteria conferring drug resistance and increased adaptation to the new niche [2, 20]. *M. abscessus* have been found in association

with free living amoebae; Delafont and collaborators [9] described the presence of NTM in over 87% of amoebae collected from drinking water networks emphasizing that NTM persist and replicate in amoebae. As NTM and amoebae share ecological niches, NTM may have gained virulence traits (i.e. from other bacteria [21]) to overcome amoebae and replicate intracellularly becoming more adapted for infection and survival in human macrophages.

In summary, *M. abscessus* subsp. *massiliense* isolates from human patients are cytotoxic to *A. castellanii*. While early infection stage smooth strains showed faster growth compared to late infection rough counterparts, the latter were more cytotoxic. The rough morphotype certainly plays a role in increasing amoeba cell death; however, other cytotoxic factors might be involved. The interactions between NTM and amoebae have gained importance in recent years due to their co-occurrence in nature [9], which suggests that amoebae may serve as a reservoir for mycobacteria. In fact, all 4 strains included in this study were able to replicate within *A. castellanii* trophozoites and remain viable within cysts. *A. castellanii* is a useful experimental model to study host-pathogen interactions such as invasion, intracellular growth, and resistance to intracellular killing. While the amoeba model may not be suitable to study all virulent traits of *M. abscessus* in human infections, it can provide insights into pathogenic pathways from strains isolated at different stages of infection.

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Fig. 1.

Growth curves of *M. abscessus* subsp. *massiliense* B1, B8, RS1, and RS8 over the course of 72 h in *Acanthamoeba castellanii*. Smooth morphotypes B1, and RS1, show increased growth after 72 h in *A. castellanii* when compared with rough morphotypes.

Fig. 2. Colonies of *M. abscessus* subsp. *massiliense* B1 (A), B8 (B), and RS1(C), RS8 (D) showing smooth and rough mycobacteria collected in early and late stages of pulmonary infections, respectively. E and F: Black arrows indicate clumps of *M. abscessus* B8 and RS8, respectively, stained with Kinyoun after 72-h of *A. castellanii* infection. G and H: Cysts of *A. castellanii* containing *M. abscessus* B8 and RS1(black arrows) after 7-day infection (1000 X, - Bright field microscope (1000X).

Fig. 3. Percentage of *Acanthamoeba castellanii* cell death infected with *M. abscessus* subsp *massiliense* B1, B8, RS1, and RS8, measured by LDH release. Data show that mycobacteria isolated in early stages of lung infection (RS1 and B1) are less cytotoxic to *A. castellanii* than mycobacteria isolated in late stage of lung infection. Statistically significant differences, paired tailed Student's *t*-test ($P < 0.05$).