INSTITUT PASTEUR

Research in Microbiology 158 (2007) 393–397

www.elsevier.com/locate/resmic

The Neff strain of *Acanthamoeba castellanii*, a tool for testing the virulence of *Mycobacterium kansasii*

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Received 26 October 2006; accepted 22 January 2007

Available online 20 February 2007

Abstract

Virulent *Mycobacterium kansasii* (mainly subtype 1) may cause lung infections, whereas certain other strains (essentially subtype 3) are commonly non-pathogenic mycobacteria colonizing the human lower respiratory tract of patients. Determining the clinical significance of a strain isolated from a respiratory sample represents a major challenge for clinicians. Since some mycobacteria may use free-living amoebae as a training ground to select virulence traits, we wondered whether the *Acanthamoeba castellanii* amoeba could be used to determine the virulence of these intracellular bacteria. We investigated whether the growth and cytopathic effect of *M. kansasii* in *A. castellanii* correlate with the virulence of *M. kansasii* determined clinically and by subtyping.

Pathogenic subtype 1 *M. kansasii* strains grew better in *A. castellanii* than non-pathogenic subtype 3 strains when considering both the number of bacteria per amoeba and the percentage of infected amoebae. Moreover, a subtype 3 *M. kansasii* strain isolated from blood culture, and thus considered pathogenic, was revealed to grow in *A. castellanii* similarly to pathogenic subtype 1 strains.

These results suggest that amoebae may represent useful tools for testing the virulence of intracellular mycobacteria and other amoeba-resisting bacteria. This is important, since identification of novel bacterial virulence factors relies largely on in vitro assessment of virulence.

Keywords: Mycobacteria; Free-living amoeba; Virulence

1. Introduction

There are significant ethical and practical limitations to our ability to assess bacterial virulence and to determine the relative importance of each virulence factor in animal models. This has led some research groups to develop alternative host systems such as plants (*Arabidopsis* sp.) [23], insects (*Drosophila melanogaster*) [8] and worms (*Caenorhabditis elegans*) [30], in which bacterial virulence may be analyzed in a simple manner. These models are, however, best suited for the study of extracellular bacteria, since phagocytic cells are absent or poorly characterized in these organisms. *Dictyostelium discoideum* is a phagocytic amoeba that has been successfully used as an in vitro model to study the virulence of *Pseudomonas aeruginosa* [7,22] and *Legionella pneumophila* [16,28].

Acanthamoeba is a free-living amoeba that may represent an additional model to study the interaction of intracellular microorganisms with phagocytic cells. Indeed, a recent

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doi:10.1016/j.resmic.2007.01.003

study showed that *P. aeruginosa* isolated from blood cultures were more virulent in the amoebal co-culture assay than environmental strains [10]. Free-living amoebae may play a role in the selection of virulence traits and in adaptation to survive in macrophages [15]. Thus, intra-amoebal growth may morphologically modify the development of intracellular bacteria such as *L. pneumophila* [14], and this may be associated with increased virulence, as shown in HeLa cells [11].

Several mycobacteria can grow within amoebae, including *Mycobacterium bovis* [31], *Mycobacterium xenopi* [9], *Mycobacterium marinum* [27], *Mycobacterium massiliense* [2], *Mycobacterium smegmatis* [26] and *Mycobacterium avium* [19,33]. Moreover, Adekambi et al. recently showed that several species may not only resist destruction by free-living amoebae, but also survive within cysts [1]. Thus, amoebae may represent a widespread reservoir and an evolutionary crib for mycobacteria. When studying a hospital water network, mycobacteria were isolated more frequently from samples in which amoebae were present (47%) than in samples in which no amoebae were found (18%, $p = 0.009$) [32]. Moreover amoeba-grown *M. avium* was shown to be more virulent when tested in mouse models of infection than axenically-grown bacteria [6].

Among non-tuberculous mycobacteria, *Mycobacterium kansasii* is (with *M. avium*), one of the most common species responsible for human disease [12,18]. *M. kansasii* may cause lung infections in both immunocompetent patients and immunocompromised patients. However, it may also be a persistent or transient asymptomatic colonizer of the lower respiratory tract, especially in patients with bronchiectasis or chronic obstructive pulmonary disease (COPD) [29]. Determining the clinical significance of isolating *M. kansasii* from lower respiratory tracts samples represents a major challenge for clinicians. *M. kansasii* has recently been classified into different subtypes on the basis of restriction fragment length polymorphism analysis of PCR products (PCR-RFLP) of the *hsp65* gene [3,21,24,25]. Subtype 1, the most frequent *M. kansasii* isolate from human samples, is generally considered to be pathogenic, as it is isolated from patients with lung infections [17,34]. Subtype 3 should be considered as a colonizer [29], since it is mainly isolated from lower respiratory tract samples taken from patients without signs and symptoms fulfilling the American Thoracic Society criteria of lung infection [4]. These colonized patients frequently presented COPD or bronchiectasis [29]. Subtype 2 is usually considered to be an opportunistic agent, since it has been mainly isolated from HIV-infected immunocompromised patients [29]. The pathogenicity of the other subtypes (subtypes 4–7) remains to be characterized.

As free-living amoebae may represent a useful tool for determining the virulence of intracellular bacteria, we investigated whether the growth of *M. kansasii* in *Acanthamoeba castellanii* correlates with its virulence, i.e. whether pathogenic subtype 1 strains grow better than non-pathogenic subtype 3 strains.

2. Materials and methods

2.1. M. kansasii strains

Strains used for this study were clinical *M. kansasii* isolates collected in Switzerland between 1991 and 1997 [29] that have been characterized biochemically and genetically by PCR restriction enzyme pattern analysis of the *hsp65* gene [29]. A total of 14 strains of *M. kansasii* were evaluated for their growth in *A. castellanii*. These included five pathogenic subtype 1 strains, five non-pathogenic subtype 3 strains, one pathogenic subtype 3 strain and three subtype 6 strains. The pathogenicity of the strains was determined according to the American Thoracic Society criteria [4] and the source of the isolate (i.e. considered pathogenic when isolated from blood culture). After 15 days culture on Middlebrook 7H10 agar (Difco), several colonies were washed three times with PBS (Gibco-BRL). The suspension was then 5-μm-filtered to avoid mycobacterial aggregates [20]. The final suspension was adjusted to a concentration of 1×10^8 bacteria per milliliter using the McFarland turbidity standard.

2.2. Infection procedure

A. castellanii ATCC 30010 was grown axenically in 75-cm^2 culture flasks (Corning) in peptone-yeast extract glucose broth medium at 28 °C [13].

Cells were harvested and resuspended in Page's modified Neff's amoeba saline (PAS) [13] to a concentration of 1×10^5 amoebae per milliliter. Adherent *A. castellanii* (1×10^5 amoebae/well) in 24-well tissue culture plates (Corning) were infected with mycobacteria at a multiplicity of infection (MOI) of 1 (1×10^5 bacteria/well). The plates were then centrifuged at 1500*g* for 30 min before incubation at 32 °C in a moist atmosphere.

The number of bacteria per amoeba and the percentage of infected amoebae was determined by light microscopy after heat-fixation of the cells and Ziehl–Neelsen staining. To determine their viability, amoebae were harvested, diluted with an equal volume of 0.4% trypan blue (Sigma-Aldrich) and of PBS and then transferred to a Kova counting slide (Hycor Biomedical). Numbers of unstained (living) and stained (dead) amoebal trophozoites and number of cysts were determined in triplicate. The encystment rate was defined as the number of cysts divided by the total number of amoebae (cysts, living trophozoites and dead trophozoites).

2.3. Statistical analysis

Mean numbers of bacteria per amoeba, percentage of infected amoebae and number of dead amoebae were compared using the unpaired *t*-test performed with GraphPad Prism Software (Prism 4.0).

3. Results

To examine the virulence of *M. kansasii* in *A. castellanii*, we infected the amoebae with five pathogenic subtype 1