The role of free-living pathogenic amoeba in the transmission of leprosy: a proof of principle

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Summary
Objectives Leprosy transmission remains poorly understood, though, prolonged skin contact and/or infection via nasal mucosa, are considered likely. Problematic in any transmission hypothesis is the fastidious nature of Mycobacterium leprae outside its host cell and the requirement for temporary survival in the environment, soil or water. Experiments were carried out to test the hypothesis that free living pathogenic amoeba might serve as host cells for M. leprae, protecting them from adverse environmental conditions.

Design In this study we employed cultures of Acanthamoeba castellanii, a free-living pathogenic soil amoeba, to determine whether these protozoa can ingest M. leprae and whether the intracellular bacilli remain viable.

Results More than 90% of cultured amoeba ingested M. leprae at a 20:1 multiplicity of infection while the infected amoebae thrived and multiplied normally. The ingested M. leprae were not degraded and remained viable for at least 72 hours as determined by their metabolic activity (radiorespirometry) and cell wall integrity (viability staining). M. leprae isolated from infected amoebae multiplied at the same rate as freshly harvested bacilli in the foot pads of nu/nu mice.

Conclusions These findings provide proof of principle that free-living pathogenic amoeba are capable of ingesting and supporting the viability of M. leprae expelled into the environment. Studies are underway to determine whether M. leprae-infected A. castellanii and other pathogenic amoebae may also play a role in transporting leprosy bacilli through broken skin or the nasal mucosa.

Introduction

The routes, methods and mechanisms of transmission of leprosy are poorly understood. However, some of the pieces of the leprosy transmission puzzle have been partially defined and support reasonable hypotheses. For example, it seems likely that the enormous numbers of leprosy bacilli expelled into the environment in the nasal discharges of lepromatous
patients\textsuperscript{1} is one source of infection and Desikan has demonstrated detectable viability of \textit{M. leprae} in nasal discharges.\textsuperscript{2} There is also evidence to support excretion of bacilli from skin lesions.\textsuperscript{3} Other experimental evidence supports the entry of bacilli into a new host via one of two (or both) portals; invasion and subsequent infection though the nasal mucosa\textsuperscript{4} or abraded or punctured skin.\textsuperscript{5}

Regardless of the hypothetical route of transmission, there remains an enigma. How does \textit{M. leprae}, an obligate intracellular pathogen that is so fastidious, it cannot be cultured after 130 + years of effort, remain viable and infectious in a rather inhospitable environmental niche between hosts? In the present hypothesis we explore whether \textit{M. leprae} could be taken up by free-living pathogenic amoebae in the soil or water and survive, sheltered intracellularly in these protozoa serving, essentially as ‘feral macrophages’.

There is ample precedence for this hypothesis. A number of studies report that microorganisms can survive endosymbiotically in free-living pathogenic amoebae.\textsuperscript{6} In 1980 an association between \textit{Acanthamoeba} and \textit{Legionella pneumophila}\textsuperscript{7} was reported by Rowbotham who suggested that infected amoebae were the source of Legionaires Disease. Subsequently \textit{L. pneumophila} was shown to resist phagosome-lysosome fusion and multiplies in \textit{A. castellanii}\textsuperscript{8}. A number of other organisms have been shown to be endosymbionts in \textit{Acanthamoeba} spp, including \textit{Pseudomonas},\textsuperscript{9} \textit{Chlamydia},\textsuperscript{10} \textit{Burkholderia}\textsuperscript{11} and \textit{Listeria monocytogenes}.\textsuperscript{12} Several reports describe infection of \textit{Acanthamoebae} with environmental\textsuperscript{13} or pathogenic mycobacteria such as \textit{M. avium-intracellularae},\textsuperscript{14,15} \textit{Mycobacterium paratuberculosis},\textsuperscript{16} \textit{M. bovis} and \textit{BCG}.\textsuperscript{17}

Infection of amoebae with \textit{M. leprae} was suggested by Jadin in 1975 who demonstrated the uptake of \textit{M. leprae} by \textit{Acanthamoeba castellanii}\textsuperscript{18} and by Grange et al.\textsuperscript{19} in 1987. Our findings extend these reports by exploiting our routine access to highly viable and purified nu/nu-derived \textit{M. leprae}\textsuperscript{20,21} coupled with \textit{in vitro} and \textit{in vivo} assays for \textit{M. leprae} viability. We pose the question of whether the leprosy bacillus survives intracellularly in \textit{A. castellanii}.

\section*{Materials and Methods}

\textbf{A. CASTELLANII CULTURE}

Frozen \textit{Acanthamoeba castellanii} (Neff) culture was obtained from ATCC (Cat # 30010) and maintained in T75 flasks (Corning) by twice weekly passage at room temperature (26 \textdegree C) in ATCC 712 medium (www.atcc.org product information sheet 30010). \textit{A. castellanii} was also maintained in culture on a non-nutrient agar surface coated with killed \textit{Escherichia coli} as described by Neff.\textsuperscript{22} Briefly, 1·0 ml (1 \times 10^9) irradiated \textit{E. coli} (10^6 Rad [Sheppard Model 484 60Co irradiator]) was spread evenly on 80 mm Petri plates of non-nutrient agar (Difco). An \textit{A. castellanii} suspension (5 \times 10^6 amoebae in 0·1 ml) was then spotted at the centre of the plate and the cultures incubated at 33 \textdegree C. Over a 24–48 hour period the amoebae ingested the \textit{E. coli}, spread radially and multiplied.

\textbf{NUDE MOUSE DERIVED \textit{M. LEPROAE}}

The Thai-53 isolate of \textit{Mycobacterium leprae} was maintained in the foot pads of athymic nu/nu mice infected for 4–6 months, and then harvested as described previously,\textsuperscript{20} washed by centrifugation, resuspended in RPMI-1640 (Gibco) + 10\% (v/v) fetal calf serum [(FCS) Gibco] and enumerated by direct count according to Shepard’s method.\textsuperscript{23} \textit{M. leprae}
suspensions were purified by NaOH treatment as described previously. Briefly, $1 \times 10^9$ fresh \textit{M. leprae} were suspended in 1·0 ml of 0·1N NaOH (Sigma) and incubated for 3 minutes at room temperature, after which the bacteria were washed three times in Hanks balanced salt solution and finally resuspended in the appropriate media. Freshly harvested viable bacilli were always employed in experiments within 24 hours of harvest.

**STAINING OF \textit{M. LEpraE} WITH THE VITAL RED STAIN PKH26**

For confocal microscopy studies, freshly harvested and 0·1N NaOH treated \textit{M. leprae} were stained with fluorescent red PKH26 dye (Sigma) following a published protocol. Briefly, \textit{M. leprae} were stained for 2 minutes at room temperature with a 1:250 dilution of PKH26 dye. The suspension was washed three times in appropriate medium. The numbers of bacteria were recounted following staining by Shepard’s direct count method.

**INFECTION OF \textit{A. CASTELLANII} WITH LIVE \textit{M. LEpraE}**

Monolayers of \textit{A. castellanii} cultures in T75 flasks (Corning) containing 15 ml of ATCC 712 media were infected with viable \textit{M. leprae} at an MOI of 20:1 and incubated overnight at 26°C. Extracellular \textit{M. leprae} were removed by decanting the media and washing 3 times with phosphate buffered saline (PBS). For some experiments infected amoebae were removed by vigorous shaking after chilling the cultures at 4°C. For other experiments intracellular \textit{M. leprae} were released from amoebae by lysis with 0·1N NaOH (Sigma) at 24, 48, 72 and 96 hour post infection, and the \textit{M. leprae} processed for radiorespirometry, viability staining and inoculation into nu/nu mouse foot pads.

**STAINING OF INFECTED \textit{A. CASTELLANII} TROPHOZOITES**

Briefly, \textit{A. castellanii} cultures were stained with carbol fucshin for 20 minutes at room temperature. After washing with acid alcohol the slides were counter stained with methyl green.

**CONFOCAL MICROSCOPY**

A Leica SP2 confocal microscope was used to ascertain internalisation of live \textit{M. leprae} by \textit{A. castellanii} trophozoites. Serial optical sections of the infected amoeba were taken at 0·2 nm using 514 nm excitation laser and 560 ± 20 nm emission filters.

**RADIORESPIROMETRY (RR)**

Metabolism of suspensions of control and amoeba-derived \textit{M. leprae} was measured by evaluating the oxidation of $^{14}$C-palmitic acid to $^{14}$CO$_2$ by RR as described previously. Briefly, $1 \times 10^7$ \textit{M. leprae} were suspended in 4·0 ml of acidified Middlebrook 7H12 BACTEC PZA media (Becton Dickinson) in a 5 ml glass vial with loosened cap which, in turn was inserted into a wide mouth liquid scintillation vial lined with filter paper impregnated with NaOH, 2,5-diphenyloxazole (Sigma) and Concentrate I (Kodak). When read daily, captured $^{14}$CO$_2$, determines the rate of $^{13}$C-palmitic acid oxidation. In the present study the 7th day cumulative counts per minute (CPM) are reported. This measure of
metabolic activity by suspensions of *M. leprae* correlates highly with viability as demonstrated by growth in the mouse foot pad.  

**FLUORESCENT VIABILITY STAINING (VS) OF *M. LEPRAE***

The membrane integrity of amoeba-derived *M. leprae* was evaluated with a *LIVE/DEAD BacLight Bacterial Viability Kit*® (Molecular Probes) as described previously.  

Briefly, *M. leprae* were washed in normal saline and incubated for 15 minutes at room temperature with Syto9 and propidium iodide (PI). The bacteria were washed again and resuspended in 10% (v/v) glycerol in normal saline. The dead and live bacteria were enumerated by direct counting of fluorescent green and red bacilli using appropriate single bandpass filters. The excitation/emission maxima are 480 nm/500 nm for Syto9 and 490 nm/635 nm for PI. This VS method measures the cell wall integrity of individual bacilli and correlates highly, both with RR and growth in the mouse foot pad.  

**NUDE MOUSE FOOT PAD GROWTH OF *M. LEPRAE***

Athymic nu/nu mice, four in each group, were inoculated on the plantar surface of both hind feet with $5 \times 10^6$ *M. leprae* harvested 72 hour post infection from *A. castellanii* or control *M. leprae* incubated in parallel at 26 °C. At 4- and 7-months both hind foot pads were harvested from each of two mice, processed and the number of AFB enumerated using Shepard’s technique.  

**STATISTICAL ANALYSIS***

The data are shown as means ± standard deviation (SD) from a representative of three to four experiments. The raw data were subjected to Student’s *t* test to determine whether the observed differences between the means were significant. *P* < 0.05 was taken as significant.  

**Results**

**UPTAKE OF *M. LEPRAE* BY *A. CASTELLANII***

As shown by acid fast staining of infected amoebae in Figure 1A and confocal microscopy of fluorescent stained *M. leprae* in Figure 1B, the amoebae were able to efficiently ingest live *M. leprae* after overnight incubation at a MOI of 20:1. Greater than 90% of amoebae were infected (Figure 1A). Ingestion of live *M. leprae* did not show any apparent adverse effect on the amoeba as they divided normally over several days (data not shown) and did not form cysts.  

**CULTURE OF *A. CASTELLANII* ON A SURFACE CONSISTING OF *E. COLI* OR *M. LEPRAE***

Figure 2B shows progress of spreading *A. castellanii* culture at 33 °C on non-nutrient agar coated with irradiated *E. coli*, while amoeba did not advance on either non-nutrient agar alone (2A) or on non-nutrient agar coated with irradiated *M. leprae* (2C).

Similar findings were seen at an incubation temperature of 37 °C while the radial advance of amoebae on the *E. coli* plates was markedly reduced at 26 °C (data not shown). These
results indicate that *A. castellanii* was not able to thrive solely on irradiated *M. leprae* as it could efficiently on irradiated *E. coli*. 

**IN VITRO TESTS FOR VIABILITY OF M. LEPRAE ISOLATED FROM INFECTED A. CASTELLANII**

Both RR and VS results show negligible loss of viability in *M. leprae* ingested by amoebae even after 96 hour (RR $P = 0.802$, VS $P = 0.783$) (Figure 3A and 3B).

It was difficult to perform the assays beyond 96 hours due to the multiplication of the amoebae and the consequent dilution of the infected population. In a representative
experiment where 90% of the amoebae were infected at 0 hours, 49% were infected at 24 hours, 15% at 72 hours and <5% at 96 hours.

GROWTH IN THE NUDE MOUSE FOOT PAD OF M. LEPRAE ISOLATED FROM A. CASTELLANII

*M. leprae* harvested from *A. castellanii* 72 hours after ingestion were used to inoculate both hind foot pads of athymic nu/nu mice. In both groups foot pads were visibly enlarged at 4 months. At 4 and 7 months post inoculation the growth in the nude mouse foot pad of *M. leprae* harvested from *A. castellanii* 72 hour post-infection was indistinguishable from (*P* = 0.894) that of the control (Figure 4).

Discussion

Our laboratory has devoted considerable effort to defining the biophysical optima of *M. leprae*, quantifying ‘viability’ *in vitro* in this uncultivable organism and developing

![Figure 3. Radiorepirometry (A) and viability staining (B) of in vitro ingested *M. leprae* obtained by lysis of *A. castellanii* trophozoites at different time points after infection.](image)

![Figure 4. Growth of *M. leprae* in nu/nu mouse foot pad. Growth of *M. leprae* derived from *A. castellanii* 72 hr after ingestion is compared with growth of a control suspension of *M. leprae*, held at 26°C for 72 hr.](image)
routine access (weekly) to large numbers of fresh viable *M. leprae* from the foot pads of athymic (nu/nu) mice.21,24 These techniques and this valuable research resource were critical to carrying out the present study.

We chose for the present studies *Acanthamoeba castellanii* as a putative amoebic ‘host cell’ for *M. leprae* because these protozoa have been shown to harbour and support the growth of a number of other bacteria, including environmental mycobacteria;13 and there is ample precedence for *Acanthamoeba* being a host cell for several pathogenic mycobacteria; *M. avium*-intracellulare,14 *M. paratuberculosis*,16 BCG17 and *M. bovis.17* Acanthamoeba may play a role in transmission of mycobacterial infection to man.14

The present studies demonstrated the avid uptake of *M. leprae* by *A. castellanii* in a dose dependant manner. Even with a high MOI where virtually all of the amoebae phagocytosed *M. leprae*, there appeared to be no ill effect on the multiplication of the infected protozoa (data not shown). Time course observations (data not shown) revealed that with time (24–48 hours) individual intracellular bacilli, distributed throughout the cytoplasm at 0 hours, appeared to be packaged by the host amoeba into a single large vacuole.

Whereas the amoebae could survive and multiply on a simple diet of killed *E.coli*,22 *M. leprae* alone did not appear to provide the necessary nutrients and were not digested or degraded. In fact, *M. leprae* survived for at least 4 days as shown by their metabolic activity (RR) and cell wall integrity (VS), in vitro assays for viability.21 More importantly, viability after 3 days of infection of amoebae was undiminished when evaluated by inoculation into the foot pads of athymic (nu/nu) mice. We consider these findings as proof of principle supporting the hypothesis that *A. castellanii* and perhaps other soil or aquatic free-living pathogenic amoebae might perform as ‘feral macrophages’ by facilitating the survival of the leprosy bacillus in the environment when expelled from their human host.

An interesting corollary to this hypothesis would be the potential role of dormant encysted amoeba in protecting *M. leprae* during adverse environmental conditions such as dessication, changes in temperature and pH.26 In preliminary experiments with *M. leprae*-infected *A. castellanii*, where encystment was induced by raising NaCl concentration we observed, under phase microscopy, that as the trophozoites condensed in size and formed thickened cell walls they appeared to expel their particulate contents, including *M. leprae* (unpublished observation). However, a report by Steinert et al. employed electron microscopy to demonstrate the presence of *M. avium* within the outer walls of the double walled *Acanthamoeba* cyst when infected trophozoites were encouraged to encyst.15 Additional studies are being carried out with *M. leprae*.

Finally, the demonstration that *M. leprae* will survive in a pathogenic free-living amoeba will allow us to move this hypothesis forward and determine if *M. leprae* infected free-living pathogenic amoebae could play a role in the actual transmission of leprosy by facilitating the invasion of human tissue. First we will need to confirm these in vitro findings in other members of the genus *Acanthamoeba*, *A. culbertsoni*, and *A. polyphaga*, both of which have already been shown to support the growth of pathogenic mycobacteria.27 Various species of *Acanthamoeba*, *A. castellani*, *A. culbertsoni* and *A. polyphaga* are the agents of human diseases27 including Granulomatous Amoebic Encephalitis (GAE) a slowly progressive CNS infection, cutaneous acanthamoebiasis (CA), and amoebic keratitis (AK). GAE and CA are diseases seen in immunosuppressed patients while (AK) is a sight-threatening corneal disease that can occur in immunocompetent individuals and is acquired from contact lens cases or cleaning solutions. The route of infection for GAE and CA are not clearly understood but the nasal passages and/or cutaneous lesions are considered likely routes.27
In preparation for the next (in vivo) stage in these studies we will also be concerned with *Naegleria fowleri*, a notorious human pathogen.\(^2\) *N. fowleri* is the causative agent of primary amoebic meningoencephalitis, (PAM) a rare but rapidly fatal human disease.\(^2\) *N. fowleri* is found in warm fresh water ponds and lakes worldwide, inadequately chlorinated swimming pools, and moist soil.\(^2\) The portal of entry for humans appears to be the nasal mucosa with subsequent invasion of the olfactory nerve plexus and rapid travel (24 hours) of the amoebae up the olfactory nerves, through the cribiform plate and into olfactory bulb and spread to other areas of the CNS.\(^2\)

The crux of this second hypothesis is that the vast majority of infections with free-living pathogenic amoebae are of no consequence; that human disease is an extremely rare outcome. Hundreds of millions have likely been exposed to *N. fowleri* by diving, swimming or splashing in infected fresh water, yet there are less than 200 recorded cases of the rapidly fatal fulminate PAM, suggesting a high level of resistance or that infection is usually asymptomatic.\(^3\) Mice immunised with killed *N. fowleri* are highly resistant to a lethal intravenous challenge with amoebae\(^3\) and serologic studies in endemic areas show a high prevalence of protective antibodies in humans suggesting exposure without disease.\(^4\) One estimate of the probability of contracting PAM, once exposed to *Naegleria* is 1 in 100 million exposures.\(^5\)

The earliest response of the host to amoebae consists of an influx of polymorphonuclear neutrophils to the site of infection, followed by an influx of macrophages\(^5\) which have been shown *in vitro* to have a deleterious effect on target amoebae.\(^6\) This is the exact sequence of events, culminating in uptake of *M. leprae* by its preferred host cell, the macrophage that would complete the hypothesis for a role of free-living amoebae in facilitating leprosy transmission. We intend to exploit already described mouse models for *N. fowleri*\(^2,3,5–6\) and *Acanthamoeba sp.*\(^5\) infection to determine if *M. leprae* infected amoebae transport the bacilli through the nasal mucosa or through intact or abraded skin.

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**References**
