Phenotypic resistance in mycobacteria: is it because I am old or fat that I resist you?

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Objectives: We aimed to explore the phenomenon of phenotypic resistance to antimycobacterial antibiotics and to determine whether this was associated with cell age or the presence of lipid bodies.

Methods: The accumulation of lipid-body-positive [lipid-rich (LR)] cells was followed using cell staining and flow cytometry. LR cells of Mycobacterium smegmatis, Mycobacterium marinum, Mycobacterium fortuitum and Mycobacterium bovis (BCG) were separated from non-lipid-body-containing [lipid-poor (LP)] cells and their MBCs determined. We also compared the MBCs for LR and LP cells from ‘old’ and ‘young’ cultures.

Results: The LR cells of all species were more resistant to antibiotics than LP cells. For BCG, the susceptibility ratios were as follows: rifampicin, 5×; isoniazid, 16.7×; ethambutol, 5×; and ciprofloxacin, 5×. Phenotypic resistance was found in LR cells irrespective of cell age.

Conclusions: We have shown that phenotypic antibiotic resistance is associated with the presence of lipid bodies irrespective of cell age. These data have important implications for our understanding of relapse in mycobacterial infections.

Introduction

Mycobacterium tuberculosis causes chronic pulmonary infection and considerable morbidity, mortality and economic loss internationally.1,2 The currently recommended drug regimen duration is 6 months, although for >80% of patients this is too long.3 It has proved impossible to identify the patients for whom shorter regimens would be effective.4

A ‘dormant’ cell state where the bacteria are not actively dividing is often postulated to be responsible for relapse.5,6 Daniel et al.1 identify cells that are ‘lipid loaded’ and postulate they are in a dormant or quiescent state. Late stationary phase cultures of mycobacteria are known to be more resistant to drugs8 and the concentration of drug required to clear all bacteria (the MBC) rises significantly.9 Older cells express lipid bodies10–13 and it is assumed they are responsible for the phenotypic resistance found. It is uncertain whether phenotypic resistance is associated with cell age or the presence of lipid bodies. In this paper, we address this question using innovative methods to separate lipid-rich (LR) cells from lipid-poor (LP) cells.

Methods

Bacteria and culture

Isolates of Mycobacterium smegmatis (NCTC 8159), Mycobacterium fortuitum (CIP 104534), Mycobacterium marinum (M strain) and Mycobacterium bovis (BCG) (NCTC 5692) were incubated in batch cultures in Middlebrook 7H9 (Fluka) with 0.05% Tween (Sigma – Aldrich) (37°C) for all species except M. marinum, which was incubated at 30°C for the appropriate duration. Viable count was determined by a modified Miles and Misra method as described previously.14

Buoyant density separation

A 1 mL aliquot of bacterial cells was harvested from culture and washed three times by centrifugation (14 400 rpm for 3 min) with sterilized water (0.22 μm filters, Millipore). Cells were resuspended in 100 μL of 75% D2O (Sigma – Aldrich)/25% dH2O, sealed and equilibrated over 24 h without agitation. A 100 μL aliquot of cell suspension from within 1 mm of the meniscus was removed using a modified P200 pipette. Also, a 100 μL aliquot of cell suspension from within 1 mm of the bottom of the tube was removed while bubbling air to prevent cells from other layers entering the pipette tip.

Staining

Mycobacterial cells were stained with 1 mg/mL Nile red (Sigma – Aldrich) at room temperature with constant agitation for 20 min. The samples were washed with 100% ethanol and again with PBS and a 10 μL aliquot was spotted onto a clean glass slide and heat-fixed. Bacterial preparations were viewed by fluorescence microscopy at ×100 (Leica DM5500) (excitation: 480/40 nm, 540/40 nm; emission: 527/30 nm, 645/75 nm).

Flow cytometry

Flow cytometry was carried out on a Millipore Guava easyCyte™ HT. Cells were stained with Nile red as above and loaded into a round-bottomed 96-well plate. This was loaded into the flow cytometer,
which excited the samples at 488 nm and read the samples at 525/30 nm and 690/50 nm.

**Old LR cells versus young LR cells**

Samples grown to late stationary phase were designated ‘old’. Mid-exponential cultures were designated ‘young’. All samples were taken at the same timepoints relative to the growth rate. Samples were separated into LR and LP fractions and treated with each of four antibiotics: ciprofloxacin, rifampicin, isoniazid and ethambutol (all from Sigma). The antibiotics were administered at concentrations from below the MBC to >10× MBC (see Table 1). Bacteria were incubated with the drug suspended in PBS overnight (~16 h). The 96-well plates were centrifuged at 3000 rpm for 10 min and the drug-containing supernatant removed. Middlebrook 7H9 medium was then added and the plates incubated for a further 72 h. The MBC was defined as the lowest concentration that produced a sterile sample. Constant and equal inoculum sizes (300–700 cells) were maintained by on-site growth analysis in parallel.

Mixed cultures were prepared identically as above, but without the separation step. Experiments were conducted as either MBC90 or MBC trials. Results were collected with the same methodology as above.

**Results**

**Accumulation of lipid bodies**

The accumulation of cells exhibiting lipid bodies as detected by flow cytometry for all four species studied is illustrated in Figure 1. After ~100 h culture, *M. smegmatis*, *M. fortuitum* and *M. marinum* reached stationary phase, whereas BCG reached stationary phase at ~150 h. It was possible to detect lipid bodies in increasing numbers after the cultures had reached stationary phase. These data were confirmed by microscopic studies performed in parallel (data not shown). *M. smegmatis*, *M. fortuitum* and *M. marinum* all grew at approximately the same rate to approximately the same density and by 100 h contained LR cells. BCG grew more slowly but to a similar density by the time it reached stationary phase, ~150 h. Interestingly, the BCG culture seemed to contain a low level of detectable LR cells from ~120 h when there was a small plateau in the growth of the bacteria; this coincides with the first detectable LR cells in the culture and the number of LR cells rose after ~160 h.

In *M. smegmatis*, BCG and *M. marinum* cultures, ~1000 LR events were detected per 5000 events when the experiment was terminated. In *M. fortuitum* cultures, ~2000 LR events were detected. The level of LR cells began to rise earliest in the *M. marinum* culture at 76 h, followed by the *M. fortuitum* culture at 80 h and then *M. smegmatis* at 96 h. In all cultures, LR cell levels began to rise only after stationary phase had been reached. In all cases (except BCG), the level of red fluorescence did not track well with the cfu counts, seeming to peak much later than the cfu counts. In BCG, the level of red fluorescence was a good measure of bacterial biomass. In the *M. marinum* culture at the final

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**Figure 1.** Cultures of four mycobacterial species grown until levels of lipid bodies rose to detectable levels by Nile red staining compared with cfu count over time. The level of green fluorescence begins to rise after ~100 h for *M. smegmatis*, *M. fortuitum* and *M. marinum* and after ~160 h for BCG, indicating a rise in lipid body formation and a possible downshift in metabolic function. The right-hand y-axis is log cfu/mL and the left-hand y-axis is flow cytometric events (number of detected particles).
timepoint (104 h), the quantity of green fluorescence detected was greater than red fluorescence.

**Susceptibility of separated cultures**

The susceptibility of purified LR and LP cells to four antibiotics was tested in all four species. We showed that the antibiotic concentration required to sterilize a culture of LR cells was higher than that required to sterilize a culture containing the same number of LP cells (Table 1). When LR cells were analysed, there was a significant increase in the concentration of drug required to kill all of the cells.

To address whether the relative resistance demonstrated is a function of culture age or is associated with the presence of lipid bodies, we separated LR cells from young cultures and LP cells from old cultures. The MBCs for the separated old and young cultures are illustrated in Figure 2 and demonstrate that LR cells of all species and all ages require a higher concentration of antibiotic to kill them compared with LP cells whether young or old.

**Discussion**

Understanding how phenotypic resistance arises in mycobacteria is important if we are to improve treatments against tuberculosis. This is critical with recent treatment trials failing to show non-inferiority due to an excess of relapse.\(^{15}\) We addressed this question by investigating the relationship between the presence of lipid bodies in mycobacterial cells and phenotypic resistance to antibiotics.

We showed that cells from old cultures are more resistant than exponentially growing cultures as has been described previously.\(^{9,16}\) We expanded this observation significantly by demonstrating that samples with \(>95\%\) LR cells share this resistance pattern, whereas LP cells behave like the exponentially growing mixed cultures.

As in vitro mycobacterial cultures grow and age, the supernatant becomes more acidic.\(^{17}\) If left unopened, the level of oxygen available to the bacteria drops.\(^{18}\) The quantity of nutrients available to the organism will also decrease\(^{19}\) and the population density of the bacteria obviously increases. In these circumstances, bacteria are exposed to multiple stresses previously associated with the production of lipid bodies.\(^{7,10,21}\) When LR and LP cells are separated, we showed that the individual drug susceptibilities are very different. In all cases, higher drug concentrations were required to sterilize a culture of LR cells than the same number of LP cells.

Our separation technique allowed us to clarify the association of lipid bodies and phenotypic resistance. We showed that lipid bodies are not only found in old or stressed cultures; it was possible to find LR cells in young cultures. Critically important is the lack of difference between old and young LR and LP cells (Table 1). LR and LP cells reacted similarly to the drugs irrespective of whether they came from a young or an old culture.

Our observations have important implications for the treatment of mycobacterial infections and such cells are found in patients with tuberculosis.\(^{22}\) As the LR phenotype is associated with an increase in the MBC of between 3 and 40 times, these
data may come to provide the first evidence that such cells are difficult to eradicate.

It is possible that the results we obtained are influenced by the D2O separation technique. Possibly, some LR cells resuscitate and convert back to LP and some are stressed or naturally form lipid bodies during the incubation period, but similar results were achieved with samples separated using D2O and a short centrifugation step (data not shown).

In summary, we have shown that the important phenomenon of phenotypic antibiotic resistance is closely associated with the presence of lipid bodies. The relative resistance exhibited by these cell types and their presence in lung lesions provides an insight into the challenges of eradicating such cells and preventing relapse.

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**Figure 2.** A comparison of old and young LR and LP cells when separated showing that the age of the cell is irrelevant; the lipid body status is the deciding factor for antibiotic susceptibility.

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**Transparency declarations**

None to declare.

**References**
