Phenotypic resistance in mycobacteria: Is it Because I am Old or Fat that I Resist you?

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Short running title

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Key words- Antibiotic resistance, lipid rich, lipid poor, *Mycobacterium tuberculosis*.

Synopsis

Objectives

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

Methods

The accumulation of lipid body positive cells (lipid rich- LR) was followed using cell staining and flow cytometry. LR cells of *M. smegmatis, M. marinum, M. fortuitum* and BGC were separated from non-lipid body containing cells (lipid poor- LP) and their MBC determined. We also compared the MBC of LR and LP from “old” and “young” cultures.

Results

The LR cells of all species were more resistant to antibiotics than LP cells. For *Mycobacterium bovis* (BCG) the susceptibility ratios were as follows; Rifampicin-5X, isoniazid-16.7X, ethambutol-5X, ciprofloxacin-5X. 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

Mycobacteria tuberculosis (MTB) causes chronic pulmonary infection and considerable morbidity, mortality and economic loss internationally. [1,2] The currently recommended drug regimen duration is for six months, although for more than 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] Deb *et al* identify cells that are 'lipid loaded' and postulate that they are in a dormant or quiescent state. Late stationary phase cultures of mycobacteria are known to be more resistant to drugs [7] and the concentration of drug required to clear all bacteria (the MBC- minimum bactericidal concentration) rises significantly. [8] Older cells express lipid bodies [9-12] and it is assumed they are responsible for the phenotypic resistance found. It is uncertain whether phenotypic resistance is associated with cell age or with the presence of lipid bodies. In this paper we address this question using innovative methods to separate LR from LP cells.

Methods

Bacteria and culture.

Isolates of *M. smegmatis* (NCTC 8159)*, M. fortuitum* (CIP 104534)*, M. marinum* (M-strain) and *M. bovis* (BCG)(NCTC 5692) were incubated in batch cultures in sealed tubes in Middlebrook 7H9 (Fluka) with 0.05% Tween (Sigma Aldrich) (37°C for all species but *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. [13]

Buoyant density separation

An aliquot (1mL) of bacterial cells were harvested from culture and washed three times by centrifugation (20,000g for 3 minutes) with sterilise water (0.22um filters, Millipore). Cells were re-suspended in 100µL of 75% D_2O 25% dH_2O (D_2O from Sigma Aldrich), sealed and equilibrated over 24 hours without agitation. 100µL of cell suspension from within 1mm of the meniscus was removed using a modified P200 pipette. 100µL of cell from within 1mm 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 (1mg/mL) Nile Red (Sigma Aldrich) at room temperature with constant agitation for 20 minutes. The samples were washed with 100% ethanol and again with PBS and an aliquot (10µL) was spotted on to a clean glass slide and heat-fixed. Bacterial preparations were viewed by fluorescence microscopy at 100X (Leica DM5500) (excitation; 480/40, 540/40. Emission 527/30, 645/75).

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 488nm and read the samples at 525/30nm and 690/50nm.

Old Lipid-rich cells versus young lipid-rich cells

Samples grown to late stationary phase were designated 'old'. Mid exponential cultures were designated 'young'. All samples were taken at the same time points relative to growth rate. Samples were separated into LR and LP fractions and treated with each of four antibiotics; ciprofloxacin (Sigma), rifampicin (Sigma), Isonaizid (Sigma) and ethambutol (Sigma). The antibiotics were administered at concentrations from below the MBC to >10x the MBC (See Table 1). Bacteria were incubated with the drug suspended in PBS overnight (~16hours). The 96-well plates were centrifuged at 3000RPM for 10 minutes and the drug-containing supernatant removed. Middlebrook 7H9 media was then added and the plates were incubated for a further 72 hours. 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 MBC_{90} 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 was illustrated for all four species studied in Figure 1. After approximately 100 hours in culture *M. smegmatis, M.* fortuitum and *M. marinum* reached stationary phase whereas BCG reached stationary phase at approximately 150 hours. It was possible to detect lipid bodies in increasing numbers after these cultures had reached stationary phase. These data are confirmed by microscopic studies performed in parallell (data not shown). *M. smegmatis, M. fortuitum* and *M. marinum* all grew at approximately the same speed to approximately the same density and by 100 hours contained LR cells. BCG grew more slowly but to a similar density by the time it reached stationary phase, approximately 150 hours. Interestingly the BCG culture seemed to contain a low level of detectable LR cells from around 120 hours 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 approximately 160 hours.

In both *M. smegmatis* and BCG cultures approximately 1000 LR events were detected per 5000 events when the experiment was terminated. In *M. marinum* and *M. fortuitum* cultures, approximately 2000 LR events were detected. The level of LR cells began to rise earliest in the *M. marinum* culture at 76 hours, followed by the *M. fortuitum* culture at 80 hours and then *M. smegmatis* at 96 hours. In all cultures LR cell levels began to rise only after stationary phase had been reached. In all cases (excepting 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 timpoint (104 hours) the quantity of green fluorescence detected was higher than that of the red fluorecsence.

Susceptibility of separated cultures

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

To address the question of whether the relative resistance demonstrated is a function of culture age or is associated with the presence of lipid bodies we separated LR from young cultures and LP cells from old cultures. The MBCs for the separated old and young cultures are illustrated in in figure 2 and demonstrate that, LR cells of all species and of all ages require a higher concentration of antibiotic to kill them compared with the 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. [14] We have addressed this question by investigating the relationship between the presence of lipid bodies in mycobacterial cells and phenotypic resistance to antibiotics.

We have shown that cells from old cultures are more resistant than exponentially growing cultures as has been described previously. [8,15] We have 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. [16] If left unopened the level of oxygen available to the bacteria drops. [17] The quantity of nutrients available to the organism will also drop [18] 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. [19-21] When LR and LP cells are separated we have shown that the individual drug susceptibilities are very different. In all cases it requires higher concentrations of drug to sterilise a culture of LR cells than the same number of LP cells.

Our separation technique has allowed us to clarify the association of lipid bodies and phenotypic resistance. We have shown that lipid bodies are not only found in old or stressed cultures; it was possible to find LR cells in young cultures. This observation holds true for all four species tested. 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 minimal bactericidal concentration of between 3 and 40 times, it may come to provide the first evidence that such cells are difficult to eradicate.

It is possible that the results we have obtained are influenced by the D_2O 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 D_2O 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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Transparency declaration

No conflicts of interest to declare

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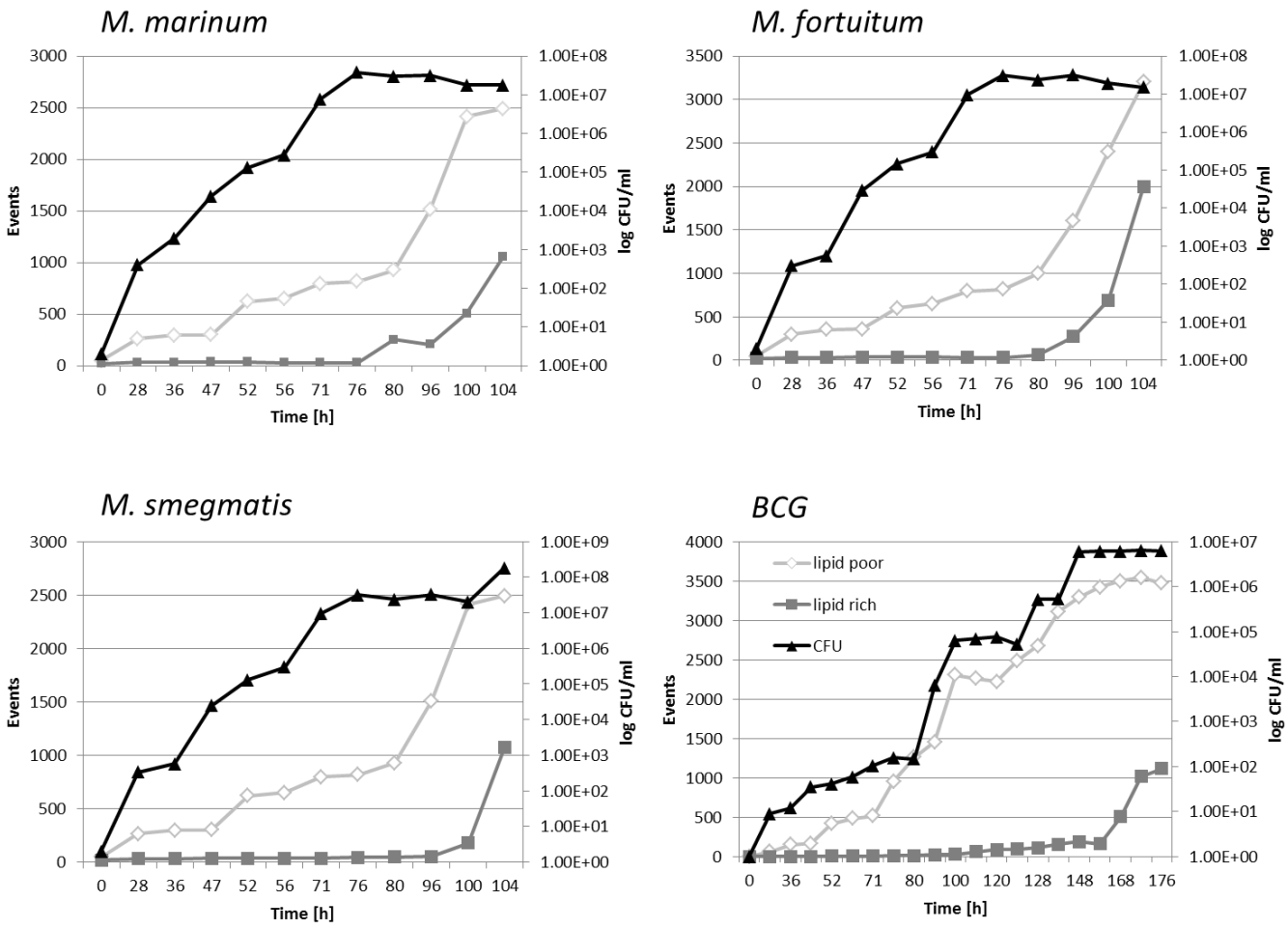

Figure 1. Cultures of four mycobacterial species grown until levels of lipid bodies rose to detectable levels resulting from Nile Red staining seen in relation to the cfu count over time. The level of green fluorescence begins to rise after ~100 hours for *M. smegmatis, M. fortuitum* and *M. marinum* and after ~160 hours for BCG indicating a rise in lipid body formation and a possible downshift in metabolic function. Right hand Y axis is Log cfu/mL and left hand Y axis is flow cytometric events; number of detected particles.

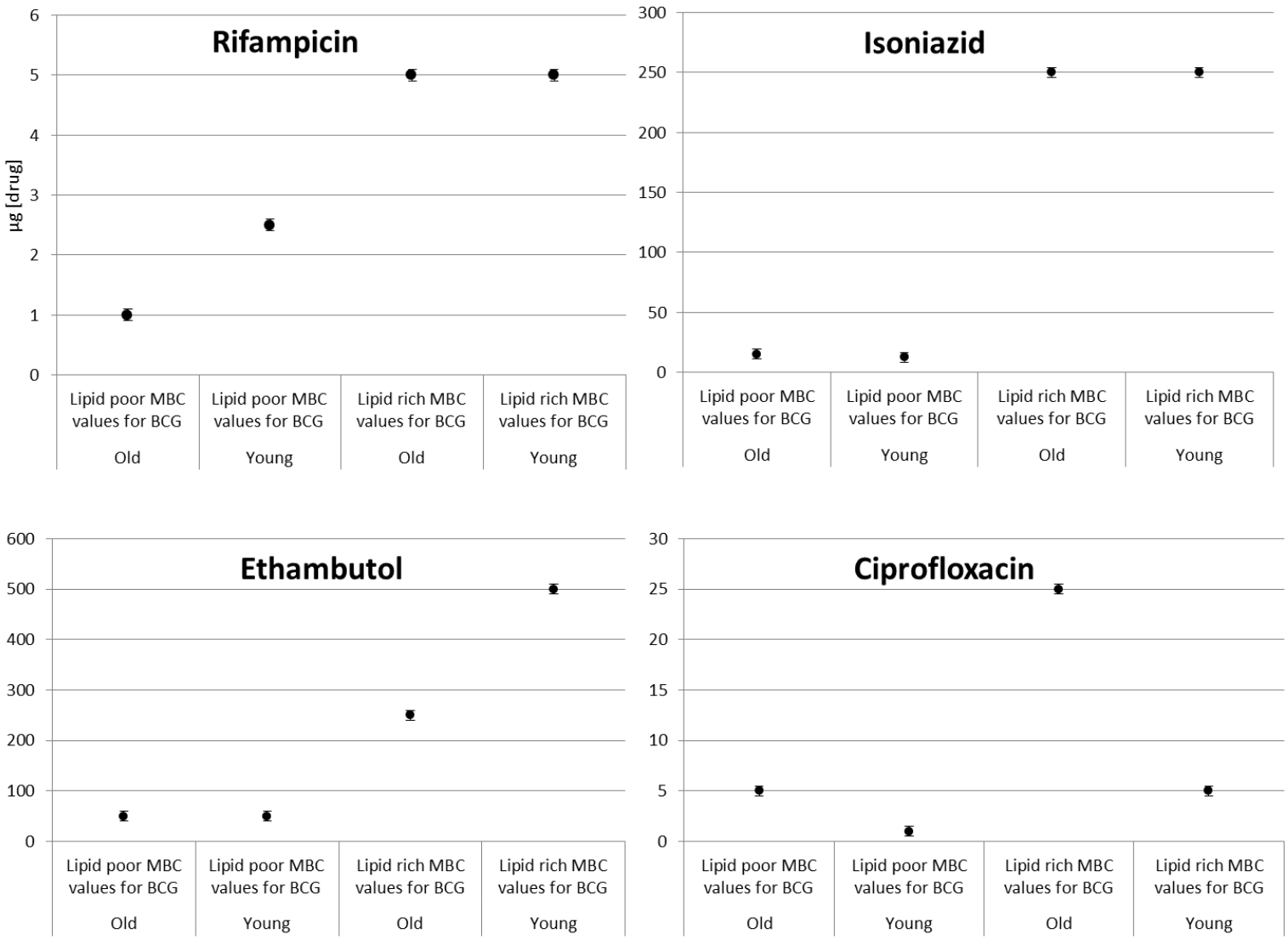

Figure 2. A comparison of old and young LR and LP cells when separated showing that the age of the cell is irrelevant, the lipid body status is the deciding factor for antibiotic susceptibility.

Drugs	LP MBC values for *M. smegmatis* (mg/L)	LR MBC values for *M. smegmatis* (mg/L)	**Increase in drug concentration required to clear LR cells (fold)**	LP MBC values for *M. fortuitum* (mg/L)	LR MBC values for *M. fortuitum* (mg/L)	**Increase in drug concentration required to clear LR cells (fold)**	LP MBC values for *M. marinum* (mg/L)	LR MBC values for *M. marinum* (mg/L)	**Increase in drug concentration required to clear LR cells (fold)**	LP MBC values for BCG (mg/L)	LR MBC values for BCG (mg/L)	**Increase in drug concentration required to clear LR cells (fold)**
Rifampicin	25	1000	**40**	50	1000	**20**	100	500	**5**	1	5	**5**
Isoniazid	30	1000	**33.3**	50	1000	**20**	50	750	**15**	15	250	**16.7**
Ethambutol	30	1000	**33.3**	na	na	na	10	150	**15**	50	250	**5**
Ciprofloxacin	35	100	**2.9**	50	250	**5**	50	250	**5**	5	25	**5**

Table 1. MBC for *M. smegmatis, M. fortuitum, M. marinum* and BCG for both LP and LR samples. LR samples required up to 40X more drug to be sterilised. Also shown; fold increases in the quantity of drug required to sterilise a culture of LR cells when compared to LP cells.