REVIEW ARTICLE

THE ROLE OF LIPID BODIES IN *MYCOBACTERIUM TUBERCULOSIS* INFECTION

***Baye Gelaw*[1]**

ABSTRACT

Lipid bodies are dynamic organelles with key roles in regulating storage turnover of lipids in different cells and organisms. Triacylglycerols (TAGs) are the dominating storage lipid in higher eukaryotes and are frequently found in eukaryotic micro organisms, like moulds, yeasts and algae. The objective of this review was to document the formation and occurrence of lipid bodies within Mycobacteria in particular and other bacteria in general. Mycobacterium tuberculosis is able to persist in the human host for decades in an apparently dormant state where it is presumed to reside in a hypoxic environment. Mycobacterium tuberculosis under stress stores triacylglycerol (TAG). There is strong evidence that fatty acids are the energy source used by the pathogen for its long-term survival during the persistence phase of infection. Triacylglycerol synthase (tgs) genes have been found to be highly induced as the pathogen enters a non-replicating state upon slow withdrawal of oxygen and upon treatment with NO. Based on the enzymatic activities of the expressed tgs gene products and the degree of their induction, tgs1 appears to have the potential to be a major contributor to TAG synthesis induced by hypoxia and NO. Along with several stress factors, such as hypoxia, nutrient deprivation, production of NO and IFN-γ by host cells, acidic pH is also considered to be one of the major stress factors which bacteria may encounter inside the host.

Sputum has been traditionally thought to contain active growing tubercle bacilli. However, recent studies rejected the commonly held belief that smear-positive sputum is dominated by aerobically replicating Mycobacterium tuberculosis. A survey on clinical samples revealed that lipid bodies were universal features of tubercle bacilli in sputum, and lipid body positive tubercle bacilli were tolerant to the bactericidal action of antibiotics and resistant to multiple stresses.

Key words: *Lipid bodies, Mycobacterium tuberculosis, Non-replicative state, Phynotipic drug tolerance, dormant tubercle bacilli.*

INTRODUCTION

In 1945 Knaysi observed the formation of fat droplets from the cytoplasm membrane in living cells of *Bacillus cereus* (1). Later, Burdon (2) confirmed the greater value of Sudan Black B dye for demonstrating intracellular fatty material in bacteria. Burdon also reported that a high proportion of gram positive bacteria were discovered to be fat stores, but many gram negative bacteria, on the other hand, were shown to be free of lipid when in active growth on common culture media.

The genus *Mycobacteria* are characterised by their high lipid content which is largely attributable to their cell envelopes (3). The cell envelope is composed of unusual lipids derived from long chain fatty acids (LCFA), and the high requirement for LCFA of these organisms is reflected by their ability to synthesise medium and long chain fatty acids via the enzyme systems, Fatty acid Synthase I and II, respectively (4). It was previously believed that the entire lipid that was synthesised or taken up by the cell was located in the cell envelope. However, recent work has revealed that lipid in the form of triacylglycerol (TAG) is also present as intracellular droplets within the *Mycobacterial* cell cytoplasm (5).

Lipid bodies have been demonstrated in tubercle bacilli in sputum obtained from a patient with tuberculosis infection, but lipid bodies have not been observed in *Mycobacterium tuberculosis* grown in the laboratory. This difference is indicative of *Mycobacterium tuberculosis* adopting an altered physiology within the host (5). Lipid bodies may store the fatty acids that supply abundance of exogenous fatty acids, suggesting that the role of *Mycobacterium* lipid bodies is more dynamic than that of a simple storage structure (6). The two most distinguishing characteristics of human tuberculosis are thought to be due to marked changes in physiology and the long incubation period of the pathogen. It is unknown whether the physiological states of the groups of bacteria underpinning these disease phenomena are equivalent or entirely discrete. Therefore, the association of

[1]*University of Gondar, College of Medicine and health Sciences, School of Biomedical and Laboratory Sciences, Department of Medical Microbiology, Gondar, Ethiopia. P.O.Box 196. Mobile 251-09-18-70-37-23. E-mail;tedybayegelaw@gmail.com*

lipid bodies in *Mycobacterium tuberculosis* with antibiotic exposure and conditions that are thought to be involved in dormancy are considered particularly relevant. The formation and occurrence of these lipid bodies within *Mycobacteria* in particular and other bacteria in general, together with the significance of lipid bodies in phenotypic drug tolerance were the focus and objective of this review.

1. Lipid storage in prokaryotes

Lipids, in addition to their role in membranes and envelopes of bacteria, can also act as storage material in prokaryotes. Many prokaryotes are able to accumulate large amounts of lipophilic compounds as inclusion bodies in the cytoplasm. The most common storage molecule is polyβ-hydroxybutrate (PHB) or other polyhydroxyalkanoates (PHAs), whereas the accumulation of triacylglycerols (TAGs) and wax esters (WEs) as intracellular lipid- bodies is a property of only a few prokaryotes (7).

Polyhydroxybutrate (PHB) is some times considered to be a carbohydrate; however, it has solubility characteristics of a lipid (8). Accumulation of PHA usually occurs in the presence of an excess of carbon source when another nutrient, such as nitrogen, phosphorous, sulphur, or oxygen is limiting (9). Polyhydroxyalkanotes (PHA) storage and metabolism is best characterized in *Ralstonia eutropha* (Figure 1), in which PHA is stored as spherical intracytoplasmic inclusions accounting for 90% of the cellular dry weight (9). Almost 150 different hydroxyalkanoic acids have now been described as constituents of PHAs (10). Accumulation of PHAs in *Ralstonia eutropha* results in the formation of approximately 10 to 20 nearly spherical intracytoplasmic inclusions per cell, with a diameter of up to 500 nm and amounting to up to 90% of the cell dry weight (11).

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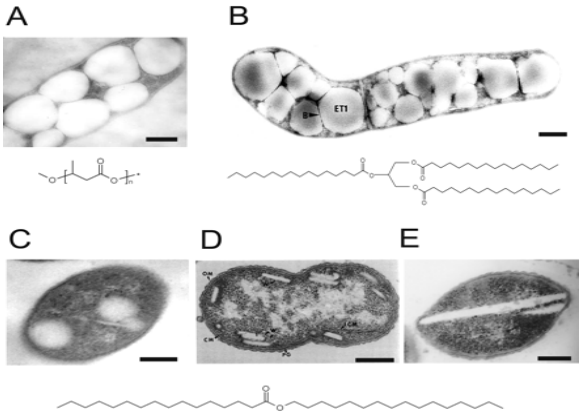

Figure 1. *Intracellular lipid inclusions in prokaryotes and general structures of the lipids. (A) Cell of R. eutropha H16 accumulating PHB inclusions; (B) cell of R. opacus PD630 accumulating large amounts of TAG inclusions; (C) cell of A. calcoaceticus ADP1 with three spherical WE inclusions; (D) Acinetobacter sp. strain HO1-N accumulating small rectangular WE inclusions; (E) Acinetobacter sp. strain M1 accumulating large, disc like WE inclusions (Adopted from Marc Waltermann and Alexander Steinbuchel, 2005).*

Triacylglycerol inclusions (TAGs) are the dominating storage lipids in higher eukaryotes and are also frequently found in eukaryotic micro-organisms, like moulds, yeasts and algae. Moreover, it has been also investigated that numerous gram-positive bacteria of the genera *Rhodococcous* (e.g. *R. opacus* and *R. rubber*), *Nocardia*, *Streptomyces* (e.g. *S. coelicolor*) and *Mycobacterium* (e.g. *M. smegmatis*) are capable of synthesizing substantial amount of TAG and deposit them as insoluble inclusions in the cytoplasm (11). Triacylglycerol (TAG) bodies were first isolated and chemically investigated from *R. opacus* strain PD630 by Alvarez et al. Triacylglycerol (TAG) bodies were mainly composed of TAGs (87%), diacylglycerols (DAGs) (5%), free fatty acids (5%), phospholipids (1.2%), and proteins (0.8%) (12). Triacylglycerols are fatty acid triesters of glycerol and represent a convenient and highly efficient form of storing fatty acids (13). Accumulation of TAG is stimulated by entry into stationary phase or upon cultivation in media with low nitrogen-to- carbon ratio. Triacylglycerol inclusions in cells grown under these conditions can almost completely fill the cell and are thought to act mainly as energy and carbon storage bodies. However, there have been a number of other functional roles suggested for TAG bodies, such as deposits for toxic or surplus fatty acids from phospholipid biosynthesis (14).

Wax esters (WE): The first report on WE biosynthesis in gram negative bacteria was published more than 30 years ago, mainly involving the genus *Acinetobacter* (15). Meanwhile, accumulations of WEs were also described for *Moraxella*, *Micrococcus*, and *Fundibacter* (16). Wax ester biosynthesis has also been reported in *Actinomycetes*, for example, in *Corynebacterium*, *M. tuberculosis* and *Nocardia* (14). In *Acinetobacter calcoaceticus*, WEs can reach a fraction of about 25% of the cellular dry weight indicating that WEs act as main storage compound(17). The shape of WE bodies is not restricted to spherical inclusions, and some authors described flat, disk-like, or rectangular inclusions when the cells were cultivated on alkanes or alkanols, respectively. The function of extra cellular WEs and the mechanisms of their export are not yet known (18). However, the main function of TAGs and WEs is to serve as a storage compound for energy and carbon. Lipid bodies may act as a deposit for toxic or useless fatty acids during growth on recalcitrant carbon sources which have to be excluded from the plasma membranes and phospholipid biosynthesis (19).

2. Formation of prokaryotic lipid bodies

The gram negative bacterium *Acinetobacter calcoaceticus* ADP1 accumulates minor amounts of TAG in addition to wax esters. Recently, the key enzyme of storage lipid biosynthesis in this bacterium was identified: the bi-functional wax ester synthase/Diacylglycerol acyltransferase (WS/DGAT). The WS/DGAT constitutes a novel, highly unspecific acyltransferase which synthesizes at the same time as wax esters and TAG (20). Under in vitro conditions, WS/DGAT shows a broad capability of utilizing a large variety of fatty alcohols and even thiols as acceptors of the acyl moieties of various acyl- CoA thioesters (21). The substrate specificities of this acyltransferases are extraordinarily broad, and it can be considered as a promiscuous enzyme. The specific activity of WS/DGAT with diacylglycerol as an acyl acceptor was one magnitude lower than with long- chain fatty alcohols, thus resembling the distribution of both lipid types in this bacterium (22). Wax ester syntase /Diacylglycerol acyltransferase (WS/DGAT) from *Acinetobacter calcoaceticus* ADP1 represents the first members of a widespread class of bacterial WE and TAG biosynthesis enzymes, because in all bacteria sequenced so far and known to accumulate neutral lipids, genes for homologous acyltransferase were detected. For example, a total of 15 *M. tuberculosis* H37Rv genes were identified and designated as triacylglycerol synthase (tgs) (23).

Three different classes of enzymes are known to mediate TAG formation from diacylglycerol (DAG) (14). Diacylglycerol acyltrnasferase (DGAT) catalyzes the acylation of DAG using acyl- CoAs as substrates. In eukaryotes, two DGAT families (DGAT1 and DGAT2) with no sequence resemblance to each other have been identified and characterized. Members of the DGAT1 gene family were found in animals and plants (24, 25), where as members of the DGAT2 gene family are found in animals, plants and *Saccharomyces cervisiae* (26, 27, 28). Acyl- CoA independent TAG synthesis in yeast and plants is mediated by phospholipids (PL): Diacylglycerolacyltransferase (DGAT) (PDAT) that uses PLs as acyl donors and DAG as an acceptor. A third alternative mechanism present in animals and plants is TAG synthesis by a DAG- DAG- transacylase, which uses DAG as an acyl donor and as an acceptor, yielding TAG and monoacylglycerol (29). *Streptomyces coelicolor* synthesizes neutral lipid storage compounds during its post exponential phase of growth in submerged liquid culture. The lipid bodies are composed mainly of TAG. No WE accumulation has been detected in this microorganism (30).

3. Lipid bodies in *Mycobacteria*

Although there have been numerous reports of the occurrence of lipid inclusions in *Mycobacteria*, the composition of the lipids in these bodies remained unknown until recently. Lipid bodies (also known as lipid droplets, adiposomes) are dynamic organelles with key roles in regulating storage turnover of lipids indifferent cells and organisms. Lipid bodies were first reported in *Mycobacteria* by Burdon (1946) by a Sudan black lipid staining method. Further light and electron microscopy studies have demonstrated the presence of these structures in a number of *Mycobacterium* species, including *M. avium* (1950), *M. leprae* (1956), *M. kansasii* (1965), *M. smegmatis* (1972) and *M. tuberculosis* (1977) (31). *Mycobacterium bovis* bacillus Calmette- Guerin (BCG) induced a does-and time dependent increases in lipid body-inducible non-membrane bound cytoplasmic lipid domain (32). Bacillus Calmette – Guerin (BCG) is reported to be capable of adapting to anaerobiosis in vitro by shifting down to a non- replicating persistent state similar to *M. tuberculosis* (33). In a study of the interactions between fluorescent lipid probes and *Mycobacteria*, it has been observed that a substantial proportion of the cell contained intracellular lipophilic inclusions (34). The principal candidate compounds were poly-β-hydroxybutrate (PHB), triacylglycerols (TAGs) and wax esters. However, *Mycobacteria* have not been reported previously to contain PHB (5).

4. Immune response to *Mycobacterium tuberculosis* and non-replicating persistence

Virulent *Mycobacterium tuberculosis* enters the host by inhalation of infectious aerosols. The pathogen replicates in the alveolar macrophages, but in a great majority of cases, the host immune defence causes bacteirostatic that leads the pathogen to go into a state of non- replicative, drug-resistant dormancy (35, 36). The macrophage is thought to be the principal location within which pathogenic *Mycobacteria* are able to survive and replicate (37). *Mycobacterium tuberculosis* has adapted strategies to survive in the naïve macrophage through mechanisms that result in the modulation of the host cell function and prevent macrophage activation by arresting the development of a localised immune response (38). *Mycobacterium tuberculosis* cells are engulfed by the macrophage within a phagosome, a membrane-bound cytoplasmic vacuole (39).The normal maturation process of the phagosome into digestive bactericidal organelle involves progressive acidification, accumulation of hydrolytic enzymes, and fusion with lysosomal compartments (39, 40). However, *Mycobacterium tuberculosis* is able to arrest the maturation of the phagosome and prevent its fusion with lysosomal compartments, thereby maintaining the pH at 6.4 (37). *Mycobacterium tuberculosis* is also able to modulate the adaptive immune response by subverting the MHC class II presentation pathway (41). The ability of *Mycobacterium tuberculosis* to avoid this response is likely to aid the survival and persistence of *Mycobacterium tuberculosis* in the macrophage as MHC class II processing, and presentation is required for the priming of $CD4^+$ cells which release the macrophage activating molecule IFN-γ (42). When the host's immune systems is weakened, the pathogen replicates, leading to active tuberculosis.

5. The Dormancy survival regulator regulon (DosR) and *Mycobacterium tuberculosis* dormancy

Current clinical models of tuberculosis postulate a three stage pathogenic sequence. Inhalation of an infectious aerosol begins the process leading to unimpeded replication of *Mycobacterium tuberculosis* in the alveolar macrophages. The onset of cell-mediated immunity causes bacteriostatic and initiates a period of bacterial persistence in the granulomatous lesions of asymptomatic hosts. Finally, in ~10% of infected hosts, declining immune competence permits the resumption of bacterial replication leading to overt disease (41). The second stage, clinical latency, is of surpassing importance for the epidemiology and control of tuberculosis. Little is known about the nature of the persistent state in vivo or the host factors that induce and maintain it. However, for a long-term survival in the murine lung, *Mycobacterium tuberculosis* expresses isocitrate lyase (43) and in vitro studies show that a persistent or dormant state can be generated by nutrient deprivation (44) and oxygen depletion (45). A number of studies have identified oxygen deprivation (hypoxia) as a potential stimulus for triggering the transition of *Mycobacterium tuberculosis* to a non-replicating persistent state analogous to latency in vivo (46). A gradual depletion of O_2 in *M. tuberculosis* caused the pathogen to reach a non-replicating persistent state that manifested drug sensitivity and structural changes suggestive of dormant state (46). Analysis of the changes in the gene expression patterns induced by hypoxia reveals a putative transcription factor, the dormancy survival regulator regulon (DosR), which is required for transcriptional activation of most of the genes known to be strongly regulated by hypoxia (47). The role of the dormancy survival regulator regulon (DosR) was initially found as the primary mediator of the hypoxic response in *Mycobacterium tuberculosis* (46). Voskuil *et al* reported that tubercle bacilli exposed to low, nontoxic concentrations of nitric oxide in vivo enter a non-replicating persistent state marked by the induction of a 48-gene regulon under the control of the DosR, suggesting that the DosR regulon may mediate the transition of these bacilli into dormancy (41). The *Mycobacterium tuberculosis* hspX gene, which is member of the DosR, encodes α-crystalin, a member of a small heat shock protein family with chaperone activity(48), which is powerfully induced under hypoxic conditions and in lung specimens obtained from patients with active tuberculosis disease (49).The recent discovery of a novel class of diacylglycerol acyl transferase enzymes in *Acinetobacter* (50) and the subsequent characterization of 15 members of this class as triacylglycerol synthase-encoding genes (tgs1-tgs15) in *Mycobacterium tuberculosis* (23) provide a biochemical basis for the presence of lipid bodies in this organism. Intriguingly, tgs1, the most active of these enzymes, is a member of the DosR regulon (51). Some of the tgs genes have been found to be highly induced as the pathogen enters a non-replicating state, upon a slow withdrawal of oxygen and upon treatment with NO (27): two conditions known to trigger induction of some of the genes potentially involved in latency /dormancy (52).

6. Nitric oxide and lipid bodies within *Mycobacterium tuberculosis*

Nitric oxide is a nonpolar gaseous molecule that is a free radical, lipophilic and relatively insoluble in water (53). Nitric oxide production in eukaryotic systems has two dominant but not unique routes: the primary production via enzymatic oxygenation of the guandine group of L-arginine to form NO and L-citrulline by NO synthase enzymes (NOS) and the secondary production by enzymatic reduction of nitrite via nitrite reductase activity of xanthine oxidase, mitochondrial cytochrome complexes, deoxyhemoglobin and some NOS isoenzymes under anoxic conditions (54). There are three well-characterized and structurally distinct NOS isoforms encoding three specific genes: NOSI (neuronal, nNOS), NOSII (hepatocyte, HE-NOS or inducible, iNOS) and NOSIII (endothelial, eNOS) (55). The overall reaction for these enzymes is the same: NADPH-dependant oxidation of L-arginine to N-hydroxy arginine and then to NO and citrulline. Endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) enzymes are calcium (Ca^{2+}) / calmodulin dependant, produce NO in short bursts and are described as "constitutive NOS" enzymes, implying that the NO so produced is required for basal physiologic organ function. In contrast, iNOS activity is not influenced by Ca^{2+} concentration but is induced by pro-inflammatory cytokines such as TNF -α, IL-1β and IFN-γ and produce large quantities of NO for as long as the enzyme is activated (56). Inducible NOS (iNOS) is transiently inducible upon cellular responses to some exogenous factors and generate NO pool in a micro molar range (57).

Environmental conditions that *Mycobacterium tuberculosis* encounters within the phagosome and the adaptive response that the organism to replicate and persist there has largely remained elusive (58). Schnappiner *et al* provide evidence that the phagosome is inhospitable habitat, poor in conventional nutrients and damaging to bacterial components. Activation of macrophages with the cytokine interferon-γ (IFN-γ) makes life in the phagosome even harsher by lowering the pH, fusing the phagosome with lysosome and stimulating the production of nitric oxide (NO), a potent antimicrobial micro molecule (59). Nitric oxide can trigger *Mycobacterium tuberculosis* to switch from active cell division to a state of non-replicating persistence. In this dormant state, the organism resists killing by external assaults, including antimicrobial drugs (41). Wayne L.G and L.G. Hayes showed that *Mycobacterium tuberculosis* has also evolved clever ways to evade the toxic effects of reactive nitrogen intermediates (RNI). In hypoxic conditions, nitrate (NO_3^-), a degradation product of NO, is reduced by the tubercle bacilli to nitrite (NO_2^-) at a rate that is significantly greater than in aerobic conditions. The induction of nitrate reductase under hypoxic conditions may serve a respiratory function in supporting the shift of the tubercle bacilli from aerobic growth state to a state of dormancy (60). In vitro, *Mycobacterium tuberculosis* formed lipid bodies after a 4-hour NO treatment, and this treated populations of bacteria was significantly more tolerant of isoniazid and rifampicin than cultures treated without NO (control groups) (31). This was supported by Garton et al's (60) report that non-replicating tubercle bacilli (lipid loaded tubercle bacilli) were tolerant to the cidal action of antibiotics and resistant to multiple stresses. Garton et al also reported that the identification of this persister-like population of tubercle bacilli in sputum presented an exciting and tractable new opportunity to investigate both treatment response to chemotherapy and the transmission of tuberculosis. The inhibition of respiration by nitric oxide (NO), which is normally produced by activated macrophages, was found to induce a gene expression pattern that was quite similar to that found under a hypoxia induced nonreplicating state. Thus, both hypoxia and inhibition of respiration by NO may induce the pathogen to go into latency (23). More recently, Daniel et al found out that *Mycobacterium tuberculosis* recovered from hypoxia (incubated under 1% O_2), 8% of the total *Mycobacterium tuberculosis* population was resistant to 5µg/ml rifampicin and about 49% was resistant to 0.8µg/ml INH (61). Thomas Schon *et al* (62) reported that in HIV negative TB patients, arginine supplementation had a significant and favourable effect on weight gain, sputum conversion, and reduction of symptom like cough. The authors concluded that that effect was likely mediated by the increased production of nitric oxide which was known to be involved in the host defence against tuberculosis (62). Boshoff and Barry also documented that *Mycobacterium tuberculosis* was likely to encounter NO in vivo, as it was released by the activated macrophages (63). On the other hand, *Mycobacterium tuberculosis* formed Lipid bodies on nitric oxide treatment as described previously and lipid loaded bacilli was tolerant to isoniazid and rifampicin. Collective evidences raise question, on arginine supplementation during tuberculosis therapy whether it increases the bactericidal activities of the anti-TB drugs. In general, the role of nitric oxide during tuberculosis infection remains undefined and needs to be confirmed by further studies.

7. Lipid metabolism in *Mycobacterium tuberculosis*

Not only are fatty acids an important component of the *Mycobacterium* cell envelope but also a growing body of evidence to suggest that *Mycobacterium tuberculosis* uses fatty acids as carbon and energy source in vivo (64). Isocitrate lyase (icl), an enzyme which has been known to be a key enzyme of the glyoxylate cycle used by organisms that live on fatty acids (65), was shown to be vital for the pathogen's persistence inside the host demonstrating the critical role of fatty acids as energy source for *Mycobacterium tuberculosis* (66). There are two *Mycobacterium tuberculosis* icl homologus, icl1 and icl2. The exact sources of fatty acid utilized by *Mycobacterium tuberculosis* are unknown; however, there are a number of possibilities (67). Fatty acids may be acquired from the lipid rich host-cell debris in mature granulomas (68). Lung surfactant is also rich in fatty acids and can be internalised by macrophages or *Mycobacterium tuberculosis* which may be able to utilize macrophage triacylglycerol stores mobilized during phagocytosis (67). Alternatively, *Mycobacterium tuberculosis* may metabolise fatty acids stored as TAG (23).The β-oxidation cycle is the principal pathway for the degradation of fatty acids in bacteria and eukaryotes. Successive rounds of β-oxidation yield acetyl-CoA that is channelled into the citric acid cycle (74). The β-oxidation cycle generates energy in the form of one molecule of Flavin adenine dinucleotide (FADH2), Nicotinamide-adenine dinucleotide (NADH) and acetyl-CoA (69). The acetyl-CoA generated in the β-oxidation of fatty acids can be directed into the glyoxylate shunt pathway. This is the first step leading to the flux of carbon into gluconeogenesis, which is the only mechanism by which the organism can acquire and conserve carbon from growth on fatty acid as the limiting carbon source (70). The key enzyme, isocitrate lyase (icl), converts isocitrate into succinate and glyoxylate, followed by the addition of acetyl-CoA to glyoxylate to form malate by malate synthase (70, 71).The glyoxylate shunt pathway thereby passes the two decarboxylation steps of the citric acid cycle (the metabolic pathway by which acetate is oxidized to generate ATP), to allow incorporation of two carbon molecules, such as acetate from β-oxidation, into the cycle (72).Therefore, intracellular pathogens may shift their primary carbon source from carbohydrate to fatty acid in the host environment (68).

8. Lipid bodies and antibiotic tolerance

Anna Hatride (unpublished work) reported that the presence of lipid bodies in *Mycobacterium smegmatis* was associated with enhanced survival of rifampicin and isoniazid treatment when compared with cells with no lipid bodies. Demonstration of lipid body formation in non-replicating cells strengthened the hypothesis that lipid bodies are associated with antibiotic tolerance because these cells are inherently drug tolerant (31,60,73).*Mycobacterium tuberculosis* is likely to encounter NO in vivo, as it is released by the activated macrophage (74) and acts as a eukaryotic signalling molecule (75). Moreover, growth arrested cells by their very nature are generally more resistant to stress (76), and the presence of intracellular lipid bodies may provide additional protection against environmental pressures. There are instances in which lipid bodies formation appears to increase survival in conditions that may be encountered upon transmission or confer an advantage in virulence. Lipid body accumulation in *Rhodococcus* has been shown to enhance the survival of the organism on desiccation, perhaps through the oxidation of hydrocarbon, which would release water to be utilised by the cells (14). *Mycobacterium tuberculosis* may be subjected to a period of desiccation following transmission from the host and therefore, the presence of an internal reservoir of water would confer a selective advantage (40). Indeed, *Mycobacterium tuberculosis* grown under conditions of low oxygen, shown to contain a population of lipid body positive cells, was more infectious to guinea pigs than aerobically grown cultures (77). In addition, it has been suggested that the global distribution of strains of *Mycobacterium tuberculosis* belonging to the Beijing lineage may be related to different gene expressions (78). It is also reported that expression of tgs1 is up-regulated in cells belonging to the Beijing lineage (79). Therefore, it is possible to speculate that lipid body formation in these strains may contribute to the high transmissibility of this group of pathogens.

9. Accumulation of lipid bodies and loss of acid-fastness of *Mycobacterium tuberculosis*

Lipid bodies can be stained with fluorescent dyes, such as Auramine O/Nile red stain, Auramine O/lipid Tox stain, Oile red O stain, and/or Sudan black stain and viewed under fluorescence microscopy (Figure 3).

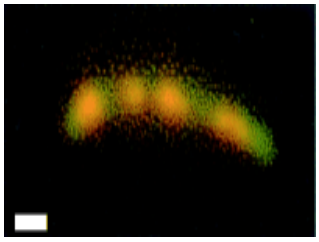

Figure 3. *Image of an acid fast cell containing the intracellular lipid inclusions in sputum sample from a patient with clinical tuberculosis. The cell is dual-labelled with Auramine O and Nile-red. Bar 2µm (Adopted from Natalie J. Garton et al, 2002).*

Studies on lipid-loaded acid fast bacilli showed a significant proportion of either poorly acid-fast bacilli or TB like nonacid fast bacilli. Andrew Bell reported that in one occasion Auramine O staining was applied, and only small proportion of *Mycobacterium tuberculosis* (less than 20%) were acid-alcohol fast (80). Moreover, Deb et al demonstrated that when a young, synchronous culture of *Mycobacterium tuberculosis* was subjected to the multiple-stress condition for increasing periods of time, a steady decrease in Auramine O stained green-fluorescing acid-fast cells with a corresponding increase in Nile red stained red-fluorescing lipid-body containing cells were reported. In that experiment, initially, in the freshly grown starter culture about 90% of the population was acid-fast positive and after 18 days under multiple-stress, acid-fast positive cells decreased to about 30% of the population while Nile red stained cells with lipid bodies increased from 10% to 70% (81). The investigators documented that the difference in dual staining property indicated the generation of at least three different sub-population in the *Mycobacterium tuberculosis* cultures under multiple-stress conditions: a subset that stained only with Auramine O (probably actively multiplying), a second subset which stained with both Auramine O and Nile red (probably transitioning to non-replicating state) and a third subset that stained only with Nile red (probably non-replicating and dormant). In addition, standard bacteriology tells us that non-replicating bacterial cells take longer to initiate growth than their replicating counterparts (longer lag phase) (82, 83).

CONCLUSION

Sputum has been traditionally thought to contain active growing tubercle bacilli. However, recent studies rejected the commonly held belief that smear-positive sputum is dominated by aerobically replicating *Mycobacterium tuberculosis.*

Surveys on clinical samples revealed that lipid bodies are universal features of tubercle bacilli in sputum. A number of conditions including hypoxia, Nitric oxide (NO) exposure, PH, heat and cold shock were shown to promote lipid body formation in *Mycobacterium tuberculosis* in vitro. The formation of lipid bodies in *Mycobacterium tuberculosis* was shown to correlate with the level of antibiotic tolerance. In addition, it has been shown that hypoxically grown *Mycobacterium tuberculosis* cultures which were lipid body-positive were 10-fold more infectious for guinea pigs than their aerobically grown counterparts. Furthermore, loss of acid-fastness following lipid body accumulation is another issue in the diagnosis of tuberculosis particularly in resource poor countries.

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