Mycobacterium tuberculosis Is Able To Accumulate and Utilize Cholesterol^{▽†}

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It is expected that the obligatory human pathogen *Mycobacterium tuberculosis* must adapt metabolically to the various nutrients available during its cycle of infection, persistence, and reactivation. Cholesterol, which is an important part of the mammalian cytoplasmic membrane, is a potential energy source. Here, we show that *M. tuberculosis* grown in medium containing a carbon source other than cholesterol is able to accumulate cholesterol in the free-lipid zone of its cell wall. This cholesterol accumulation decreases the permeability of the cell wall for the primary antituberculosis drug, rifampin, and partially masks the mycobacterial surface antigens. Furthermore, *M. tuberculosis* was able to grow on mineral medium supplemented with cholesterol as the sole carbon source. Targeted disruption of the Rv3537 (*kstD*) gene inhibited growth due to inactivation of the cholesterol degradation pathway, as evidenced by accumulation of the intermediate, 9-hydroxy-4-androstene-3,17-dione. Our findings that *M. tuberculosis* is able to accumulate cholesterol in the presence of alternative nutrients and use it when cholesterol is the sole carbon source in vitro may facilitate future studies into the pathophysiology of this important deadly pathogen.

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a very successful pathogen that infects one-third of the human population (21). Only 10% of primary infected individuals develop active disease during their lifetimes. Tubercle bacilli are able to persist in a dormant state, from which they may reactivate and induce the contagious disease state (13). In asymptomatic hosts, *M. tuberculosis* exists in reservoirs called granulomas, which are cellular aggregates that restrict bacterial spreading (40). Granulomas are organized collections of mature macrophages that exhibit a certain typical morphology and that arise in response to persistent intracellular pathogens (1, 4). Pathogenic mycobacteria can induce the formation of foamy macrophages filled with lipid-containing bodies; these have been postulated to act as a secure, nutrient-rich reservoir for tubercle bacilli (31). Moreover, *M. tuberculosis* DNA has been detected in fatty tissues surrounding the kidneys, as well as those of the stomach, lymph nodes, heart, and skin. Tubercle bacilli are able to enter adipocytes, where they accumulate within intracytoplasmic lipid inclusions and survive in a non-replicating state (26). In vivo, it is expected that *M. tuberculosis* adapts metabolically to nutrient-poor conditions characterized by glucose deficiency and an abundance of fatty acids (25, 26). The presence of a complex repertoire of lipid metabolism genes in the genome of *M. tuberculosis* suggests that lipids, including steroids, are important alternative carbon and energy sources for this pathogen (7).

One attractive potential alternative nutrient that is readily available in the mammalian host is cholesterol, a major sterol of the plasma membrane. The presence of cholesterol in lipid

rafts is required in order for microorganisms to enter the intracellular compartment (14). Studies have shown that cholesterol is essential for the uptake of mycobacteria by macrophages, and it has been found to accumulate at the site of *M. tuberculosis* entry (2, 12, 30). Moreover, cholesterol depletion overcomes the phagosome maturation block experienced by *Mycobacterium avium*-infected macrophages (10).

It is well known that cholesterol can be utilized by fast-growing, nonpathogenic mycobacteria (5, 20, 22), but it was previously thought that pathogenic mycobacteria might not be able to use cholesterol as a carbon and energy source (3). Recently, however, bioinformatic analysis identified a cassette of cholesterol catabolism genes in actinomycetes, including the *M. tuberculosis* complex (41). Microarray analysis of *Rhodococcus* sp. grown in the presence of cholesterol revealed the up-regulation of 572 genes, most of which fell within six clearly discernible clusters (41). Most of the identified genes had significant homology to known steroid degradation genes from other organisms and were distributed within a single 51-gene cluster that appears to be very similar to a cluster present in the genome of *M. tuberculosis* (41). Many of the cholesterol-induced genes had been previously selected by transposon site hybridization analysis of genes that are essential for survival of tubercle bacilli (33) and/or are upregulated in gamma interferon-activated macrophages (37, 42). It was also demonstrated that the *M. tuberculosis* complex can grow on mineral medium with cholesterol as a primary source of carbon (27, 41). Moreover, the growth of tubercle bacilli on cholesterol was significantly affected by knockout of the *mce4* gene, which encodes an ABC transporter responsible for cholesterol uptake (24, 27). Earlier studies had shown that disruption of *mce4* attenuated bacterial growth in the spleens of infected animals that had developed adaptive immunity (17, 35).

In the present study, we demonstrate for the first time that *M. tuberculosis* utilizes cholesterol via the 4-androstene-3,17-dione/1,4-androstadiene-3,17-dione pathway (AD/ADD) and

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that this process requires production of an intact KstD enzyme. We also show that tubercle bacilli growing in medium containing an alternative carbon source can accumulate cholesterol in the free-lipid zone of their cell walls, and this accumulation affects cell wall permeability.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. tuberculosis* strain H37Rv was maintained on Middlebrook 7H10 agar or 7H9 broth supplemented with 10% OADC enrichment (oleic acid, albumin, dextrose, catalase) and 25 µg/ml kanamycin (Km) when required. For growth on defined carbon sources, strains were grown in minimal medium supplemented with 0.01% cholesterol, as described previously (27).

Cholesterol uptake assay. Cholesterol uptake by *M. tuberculosis* was monitored on 7H9-OADC medium in cultures of living and thermally killed mycobacterial cells. Tritiated 1 α ,2 α (n)-[³H]cholesterol (activity of 35 to 50 Ci/mmol; Amersham Biosciences, United Kingdom) was added to the culture medium at a final concentration of 1 µCi/ml. During bacterial growth, 1-ml culture samples were taken at the indicated times (see Fig. 1) (two samples per time point) and centrifuged at 16,000 $\times$ g for 15 min at 4°C. The cell pellets were washed twice and resuspended in Tris-EDTA buffer. Cells from the first sample were mixed with OptiPhase scintillation fluid (Perkin Elmer), and the mycobacterial cell-associated radioactivity was determined by liquid scintillation counting, using a 1450 Microbeta Plus Liquid Scintillation Counter (Perkin Elmer). The second cell pellet was disrupted using a Mini Beatbeater-8 (BioSpec Products), and the resulting sample was centrifuged at 16,000 $\times$ g for 15 min at 4°C. The emissions of the supernatant (cytosolic fraction) and pellet (cell wall fraction) were monitored as described above. The activity of each sample (as nCi) was calculated from the counts per minute, after correction for counter efficiency, using an online calculator (www4.gelifesciences.com).

Filipin staining. In order to visualize cholesterol accumulation in the mycobacterial cells, we used the fluorescent dye filipin (Sigma-Aldrich). Filipin is a polyene macrolide antibiotic from *Streptomyces filipinensis* that binds specifically to cholesterol molecules; this binding causes a conformation change and emission of fluorescence. *M. tuberculosis* was cultured in 7H9-OADC medium with and without (background control) 0.01% cholesterol. After 72 h of incubation, samples from both cultures were centrifuged (16,000 $\times$ g for 15 min at 4°C), washed three times in Dulbecco's phosphate-buffered saline (PBS; pH 7.4), and fixed with 3% paraformaldehyde for 1 h at room temperature. The samples were then washed with PBS, and the paraformaldehyde was quenched by incubation with glycine (1.5 mg/ml in PBS) for 10 min at room temperature. The cells were stained with 0.05 mg/ml of filipin working solution in PBS for 45 min at room temperature in the dark, followed by three rinses with PBS and visualization by fluorescence microscopy using a Nikon Eclipse TE2000-U inverted microscope with a UV filter set (340- to 380-nm excitation; 430-nm long pass filter).

Monitoring of cholesterol uptake by gas chromatography and thin-layer chromatography. Tubercle bacilli were grown in 7H9-OADC medium supplemented with cholesterol (0.01%). To determine the accumulation of cholesterol in mycobacterial cells, 5-ml culture samples were withdrawn at 24-h intervals. The bacterial cells were spun down, washed five times for removal of extracellular cholesterol, and extracted three times with an equal volume of chloroform. To quantify the accumulation of cholesterol, equal amounts of 4-androstene-3,11,17-trione (Sigma) were added to each sample as an internal standard, and samples were subjected to gas chromatography as previously described (5). To obtain samples of the *M. tuberculosis* cell wall free-lipid zone and defatted cells, pellets were obtained from 20 ml of *M. tuberculosis* culture, washed five times, and extracted three times with an equal volume of chloroform-methanol (2:1, vol/vol) for 48 h at room temperature on a rotatory shaker (200 rpm). The resulting mixture was centrifuged at 3,200 $\times$ g for 30 min at 4°C. The resulting pellet was composed by defatted cells. The extracts were combined and evaporated to dryness under nitrogen, and the obtained free lipids and defatted cells were separated on Merck silica gel 60 thin-layer chromatography plates using chloroform-methanol-water (65:25:4, vol/vol/vol) as a solvent. The positions of the separated compounds were detected by spraying the plates with a 10% ethanolic solution of molybdophosphoric acid, followed by heating for 10 min at 180°C. Postculture medium (20 ml) was filtered through a Synpor filter (pore diameter, 0.22 µm) and extracted three times with an equal volume of chloroform-methanol (2:1, vol/vol). The extracts were combined and evaporated to dryness under nitrogen, and lipids were analyzed by thin-layer chromatography, as previously described (19).

Cholesterol biotransformation. *M. tuberculosis* cells (wild type and Δ kstD mutant) were cultured in minimal medium supplemented with 0.01% cholesterol (27). To follow the process of cholesterol biotransformation and detect intermediates, 5-ml samples were withdrawn from the culture at 24-h intervals and extracted three times with an equal volume of chloroform. The extracts were dried under a vacuum, the residue was dissolved in 0.5 ml of acetone, and the isolated steroids were analyzed by gas chromatography as previously described (34).

Cell wall permeability test. Tritiated rifampin (4-methylpiperazine-³H; activity, 10 Ci/mmol; Moravek Biochemicals) was used to examine the cell wall permeability of *M. tuberculosis* cells, based on the protocol of Piddock et al. (32). Mycobacterial cells were grown to mid-logarithmic phase (A_{600} of 1 to 1.2) on 7H9-OADC medium with and without 200 mg/liter of cholesterol. Fifty milliliters of the culture was centrifuged at 6,010 $\times$ g for 15 min at 4°C. The cells were washed in 10 ml of 50 mM sodium phosphate buffer (pH 7), resuspended in the same buffer to an optical density at 600 nm of 8, and placed in a 37°C water bath for 10 min to equilibrate. The [³H]rifampin was added at a final concentration of 0.272 µg/ml (3.33 µCi/ml), and 500-µl samples were removed at various time intervals. The samples were mixed with 1 ml of 50 mM sodium phosphate buffer (pH 7) on ice and centrifuged at 16,000 $\times$ g for 15 min at 4°C. The resulting cell pellets were washed again in the same buffer, recentrifuged, and mixed with OptiPhase scintillation fluid (Perkin Elmer). The cell-associated radioactivity was determined by liquid scintillation counting, as described above. Passive adsorption of rifampin to the cell wall (background) was estimated by performing the experiments at 0°C; these results were subtracted from the values obtained at 37°C to determine the activity from rifampin that had actively accumulated in the cells.

Antibody binding assay. Fifty-milliliter samples of *M. tuberculosis* culture (1 $\times$ 10⁸ bacteria/ml) grown in 7H9 broth with or without cholesterol were spun down at 4,000 $\times$ g for 20 min at room temperature. Each mycobacterial pellet was washed once with PBS and then with PBS supplemented with 1% bovine serum albumin, and the cells were resuspended in the latter buffer to a density of 5 $\times$ 10⁹ cells/ml. The cells were incubated with specific fluorescein isothiocyanate-labeled anti-*M. tuberculosis* antibodies (final dilution, 1:50; Abcam) for 1 h at 37°C with continuous mixing. Bacterial samples incubated without antibodies served as negative controls. The optimal working dilution of antimycobacterial antibodies was determined in preliminary titration experiments. After incubation, the experimental and control samples were washed with PBS–1% bovine serum albumin and resuspended in 100 µl of the same buffer, and fluorescence was determined using a Wallac Victor 2 reader. All samples were run in quadruplicate for two independent experiments.

Gene replacement construct and disruption of the *kstD* gene. To perform unmarked deletion of the *kstD* gene from *M. tuberculosis*, we used a suicidal recombination delivery vector based on p2N1L (28). The recombination vector carried the 5' *kstD* upstream region (1,603 bp) and the first 20 bp of the *kstD* gene tagged to the 3' part of the *kstD* gene (645 bp), followed by 1,009 bp of the *kstD* downstream region. PCR products carrying 5' and 3' fragments of the gene were ligated out of frame, such that the resulting Δ kstD gene encoded a non-functional protein. The final vector (pAB30) also included the screening cassette from pGOAL17 (28). A gene replacement strategy was used to disrupt *kstD* at its native locus on the chromosome. The plasmid DNA was treated with NaOH (0.2 mM) and integrated into the *M. tuberculosis* chromosome by homologous recombination. The resulting single-crossover homologous recombinant mutant colonies were blue, Km^r, and sensitive to sucrose. The site of recombination was confirmed by PCR and Southern blot hybridization. The single-crossover strains were further processed to select for double-crossover mutants, which were white, Km^s, and resistant to sucrose (2%). PCR and Southern blot hybridization were used to distinguish between the wild-type and double-crossover mutants (see Fig. S1 in the supplemental material). To engineer the complementation construct, the *kstD* gene was amplified using *M. tuberculosis* genomic DNA as a template and cloned into the pMV261 shuttle vector (5) under the control of the *P*_{hsp} promoter. Next, the intact gene and promoter were relocated into the pMV306 integration vector. The final construct, named pMVkstD, was electroporated and integrated into the *attB* site of the *M. tuberculosis* Δ kstD genome to complement the unmarked deletion of the *kstD* wild-type gene.

RESULTS

***M. tuberculosis* is able to accumulate cholesterol.** It is well known that fast-growing mycobacteria degrade natural sterols and use them as a source of carbon and energy (5, 20, 22). However, the ability of tubercle bacilli to utilize cholesterol

FIG. 1. Accumulation of tritiated cholesterol by living and thermally killed *M. tuberculosis* (a) and its localization in cytosolic and cellular debris fractions (b). The culture medium was supplemented with 1 μ Ci/ml of tritiated cholesterol. Samples were withdrawn and processed as described in the Materials and Methods section. Data were collected from at least three independent experiments, and the results are expressed as the geometric means $\pm$ standard deviation of the radioactivity counts.

was not observed until recently (27, 41). To determine conclusively whether *M. tuberculosis* could accumulate cholesterol, we followed the fate of tritium-labeled cholesterol supplemented into bacterial cultures. *M. tuberculosis* was grown in rich (7H9-OADC) medium, and radiolabeled cholesterol was added to living cells at early log phase and to thermally killed cells. Samples were withdrawn every 24 h and monitored for potential incorporation of cholesterol into tubercle bacilli. Bacterial cells were separated by centrifugation, washed carefully, and analyzed by scintillation counting. A significant time-dependent increase of radioactivity was observed in living cells but not in dead cells, indicating that *M. tuberculosis* actively incorporated cholesterol (Fig. 1a). For identification of the preliminary destination of cholesterol in the bacilli, cells were mechanically disrupted, and cytosolic and cellular debris fractions were analyzed. The majority of the observed radioactivity was detected in the insoluble fraction containing cell wall fragments (Fig. 1b).

The cholesterol of the mammalian cell membrane can be visualized by the fluorescent dye filipin (11, 12, 27). Accord-

ingly, we used this cholesterol-binding compound to label any cholesterol incorporated into mycobacteria. *M. tuberculosis* cells grown in the presence and absence of cholesterol were subjected to filipin staining, as described in the Materials and Methods section. Microscopic analysis revealed filipin binding of cells grown in the presence of cholesterol but not in control cells (Fig. 2). Analysis of individual bacilli indicated that the dye-bound cholesterol was deposited in the cell envelope, not intracellularly. Moreover, tubercle bacilli growing in the presence of cholesterol were treated with organic compounds to extract free lipids, the external layer of the cell wall. The defatted cells were stained in the presence of filipin and analyzed by microscopy. The dramatic decrease in fluorescence was observed by comparing defatted and control cells (Fig. 2).

As a more accurate way to identify cholesterol incorporation into bacteria, we next applied gas chromatography. Cells grown in the presence of cholesterol were collected at different time points and carefully washed, and steroids were organically extracted from these cells and analyzed in the presence of an internal standard. Our results revealed a time-dependent accumulation of cholesterol in tubercle bacilli, verifying the ability of *M. tuberculosis* grown in rich medium to accumulate cholesterol (Fig. 3A). The above data showed that tubercle bacilli are able to store cholesterol, at least when grown in rich medium, and indicated that the cell wall is a potential site of cholesterol accumulation. We hypothesized that cholesterol could accumulate in the most external layer of the cell wall, the free-lipid zone, which is more loosely formed than the other parts of the mycobacterial cell wall. To verify this hypothesis, we isolated and carefully washed cells grown in the presence of cholesterol (or tritium-labeled cholesterol), and subjected them to extraction of the free-lipid zone. The obtained extracts and defatted cells were analyzed by thin-layer chromatography, which revealed that cholesterol was, indeed, deposited in the free-lipid zone, together with phospholipids, glycolipids, and sphingolipids (Fig. 3B). The study of bacilli growing in the presence of tritium-labeled cholesterol revealed significant radioactivity in the free-lipid extracts but not in the defatted cells (see Fig. S2 in the supplemental material).

Cholesterol accumulation changes the capabilities of the mycobacterial cell wall. Having confirmed through multiple methods that *M. tuberculosis* cells can accumulate cholesterol in their cell walls, we next examined whether this ability has physiological consequences for the deadly pathogen. We hypothesized that accumulation of cholesterol might protect tubercle bacilli against toxic compounds by decreasing their cell wall permeability. To test this hypothesis, we used the first-line antituberculosis drug rifampin, which is administered to tuberculosis patients worldwide. A scintillation counter was utilized to monitor the uptake of tritiated rifampin by *M. tuberculosis* cells grown in the presence or absence of cholesterol. The obtained results clearly showed that accumulation of cholesterol by tubercle bacilli affected cell wall permeability, resulting in decreased uptake of rifampin (Fig. 4).

We also questioned whether the accumulation of cholesterol in the outer part of the cell wall could mask the surface antigens of tubercle bacilli. Using fluorescein isothiocyanate-labeled antibodies, we quantitatively examined whether the recognition of mycobacterial antigens differed between cells grown in the presence and absence of cholesterol and found

FIG. 2. Filipin staining of *M. tuberculosis* grown with (A) or without (B) cholesterol and *M. tuberculosis* defatted cells grown in the presence of cholesterol (C). Cell morphology was visualized by differential interference contrast microscopy (left). Fluorescence imaging of the same view (right) was used to examine filipin staining of cell-associated cholesterol (blue).

that bacilli grown in the presence of cholesterol showed ~20% less specific antibody binding than an equal number of *M. tuberculosis* cells cultured without cholesterol (see Fig. S3 in the supplemental material).

Cholesterol utilization requires intact KstD. A recent study using cholesterol radiolabeled with ^{14}C at the 4 position of the A sterol ring showed that the labeled carbon was converted to CO_2 when the cholesterol was added to *M. tuberculosis* culture and that this process was dependent on the Mce4 cholesterol transporter (27). We therefore wondered if utilization of cholesterol by *M. tuberculosis* could be carried out by the AD/ADD pathway and if 3-ketosteroid Δ^1 -dehydrogenase (KstD) is essential for this process. We reported previously that KstD is essential for cholesterol utilization by *Mycobacterium smeg-*

matidis and that *M. smegmatis* ΔkstD can be efficiently complemented with the *kstD* of *M. tuberculosis* delivered via a plasmid (5). More recently, KstD of *M. tuberculosis* was expressed in *Escherichia coli*, purified, and analyzed biochemically (18). The two-step recombination protocol of Parish and Stoker (28) was used to delete the *kstD* gene from the *M. tuberculosis* chromosome, as described in the Materials and Methods section. The resulting mutant was verified by PCR and Southern blot hybridization (see Fig. S1 in the supplemental material). The growth of the engineered strain in rich medium was not significantly different from that of the wild-type strain. To determine whether KstD is essential for cholesterol utilization, we compared the ability of wild-type *M. tuberculosis*, the ΔkstD mutant, and the ΔkstD mutant complemented with an intact

FIG. 3. Monitoring of cholesterol accumulation in *M. tuberculosis* cells using gas chromatography (A) and in the cell wall free-lipid zone and defatted cells using thin-layer chromatography (B). (A) The selected time points are shown on the figure. Equal amounts of the internal standard (4-androstene-3,11,17-trione) were added to each sample. Cholesterol accumulation was monitored as the ratio between the internal standard and the cholesterol peaks. (B) The bands represent the cholesterol standard (lanes 1 and 6), free lipids isolated from tubercle bacilli grown in the presence of cholesterol (lanes 2 and 7), free lipids isolated from postculture cholesterol-containing medium (lane 3), free lipids isolated from tubercle bacilli grown without cholesterol (control; lane 4), free lipids isolated from postculture control medium (lane 5), and defatted cells grown in the presence of cholesterol (lane 8). The band corresponding to cholesterol is indicated by an arrow.

kstD gene controlled by the heat shock protein promoter ($\Delta kstD$ - P_{hsp} *kstD*) to grow using cholesterol as the sole source of carbon and energy. We found that *M. tuberculosis*, but not its $\Delta kstD$ mutant, was able to use cholesterol as a primary source of carbon (Fig. 5A). As we reported previously, the unmarked deletion of *kstD* in *M. smegmatis* resulted in the accumulation of the cholesterol degradation intermediates, AD and 9-hydroxy-4-androstene-3,17-dione (9OHAD), which could not be further degraded by the mutant lacking intact KstD (5). Consistent with our findings in *M. smegmatis*, we observed that *M. tuberculosis* $\Delta kstD$ mutants grown on mineral medium supplemented with cholesterol as the sole carbon source accumulated 9OHAD in a time-dependent manner, whereas this intermediate was not observed in wild-type and $\Delta kstD$ - P_{hsp} *kstD* cultures grown in the same medium (Fig. 5B). Moreover, the time-dependent

accumulation of 9OHAD was affected by supplementation of glycerol as an alternative carbon source (Fig. 5C). Both the decreased growth of the $\Delta kstD$ mutant and its accumulation of 9OHAD on mineral medium supplemented with cholesterol are consistent with a previous report (27) that *M. tuberculosis* can utilize cholesterol as a carbon and energy source. Moreover, we also found direct evidence that cholesterol degradation in *M. tuberculosis* is performed exclusively by AD/ADD intermediates, with KstD playing an essential role in this process.

DISCUSSION

Cholesterol is an important membrane component in mammalian cells, where it plays well-documented roles in structure, signaling, and trafficking (15, 16, 23, 38). We herein demonstrate that *M. tuberculosis* can both accumulate and utilize cholesterol, depending on nutrient availability. Moreover, we found that cholesterol accumulation can change the cell wall permeability of the bacillus, and cholesterol utilization requires an intact KstD enzyme. The abilities of *M. tuberculosis* identified here by in vitro study cannot be directly applied to a discussion of the pathogenic process; however, our findings join a growing body of evidence suggesting that cholesterol may play a role in the pathogenesis of tuberculosis. Soon after inhalation, tubercle bacilli are phagocytosed by alveolar macrophages. The uptake of mycobacteria depends on the presence of cholesterol within plasma membrane lipids rafts, which accumulate at the site of mycobacterial entry (12, 30). Pathogenic mycobacteria are able to survive in the phagosomes of their host macrophages, which do not fuse with lysosomes due to maturation inhibition (8, 39). It has been postulated that the blockade of phagosome maturation requires direct contact of the phagosome membrane with the entire mycobacterial surface (9). Depletion of cholesterol from phagosomes infected with *M. avium* has been shown to lift the inhibition of maturation and allow phago-lysosome fusion (9). Thus, cholesterol binding seems to be crucial during the phagocytosis and intra-

FIG. 4. The influence of cholesterol accumulation on cell wall permeability. The uptake of tritiated rifampin by *M. tuberculosis* (M.tb) grown with (+) and without (–) cholesterol (chol) was monitored. The results were normalized by subtracting the passive adsorption of rifampin determined at 0°C.

FIG. 5. KstD is essential for cholesterol utilization. (A) Growth of *M. tuberculosis* (circles), the Δ *kstD* mutant (triangles), and the complemented Δ *kstD* strain (Δ *kstD*+*kstD*; squares) on mineral medium supplemented with cholesterol (open symbols) or untreated (closed symbols). (B) Cholesterol utilization requires an intact *kstD* gene. Gas chromatography was used to monitor 9OHAD accumulation in cultures of *M. tuberculosis* Δ *kstD* mutants grown on mineral medium supplemented with cholesterol. The 9OHAD peak was identified by simultaneous analysis of the investigated sample and a standard. No 9OHAD was detected in the wild-type and complemented mutant cultures (data not shown). (C) Cholesterol utilization is affected by the presence of alternative carbon source. Gas chromatography was used to monitor 9OHAD accumulation in cultures of *M. tuberculosis* Δ *kstD* mutants grown on mineral medium supplemented with cholesterol and glycerol (a) or cholesterol only (b). The 24-h delay in 9OHAD accumulation was observed when glycerol was present in the medium.

cellular survival of *M. tuberculosis*. The accumulation of cholesterol at these steps of infection would give tubercle bacilli an advantage within the host. As we have presented herein, cholesterol accumulation changed the cell wall permeability of the bacillus, inhibiting the in vitro uptake of the toxic compound, rifampin (a major antituberculosis drug). Moreover, cholesterol accumulation at least partially masked surface antigens in vitro, suggesting that it could help shield mycobacteria from the host immune system.

Macrophages that are infected with *M. tuberculosis* and prove unable to kill the intracellular pathogen will mature and aggregate to form granulomas containing lymphocytes, extracellular matrix components, calcifications, and caseous necrosis, which confine and eradicate the majority of tubercle bacilli (1, 31, 36). However, some bacilli are able to survive within the granuloma, resulting in a latent infection that can last the lifetime of an infected individual and may later reactivate as active tuberculosis.

It is not yet known whether cholesterol as a carbon and energy source could support long-term persistence of tubercle bacilli. As recently identified, *M. tuberculosis* possesses all the genes required to catabolize cholesterol to CO₂ via the tricarboxylic acid cycle (41). Moreover, many of these genes, including *kstD*, appear to be inducible by cholesterol (41) and essential for survival of the bacillus in the macrophage (33, 29) and in vivo in mice (35), as identified by genome-wide screening. Mutations in the *mce4* (cholesterol transporter) and *choD* (cholesterol oxidase) genes were found to attenuate *M. tuberculosis* in macrophages and in an in vivo mouse infection model (6, 27). Very recently, the *hsaC* gene (the iron-dependent extradiol dioxygenase responsible for opening of a ring A in cholesterol degradation) was found to attenuate *M. tuberculosis* in immunocompromised SCID mice and guinea pigs (43). Collectively, these findings seem to indicate that cholesterol transportation and utilization may be crucial to the fate of mycobacteria during the infection process. Here, we have directly shown that cholesterol ring structure degradation in *M. tuberculosis* occurs via the AD/ADD pathway, and disruption of *kstD* inhibits this process, leading to accumulation of intermediates. The requirement of *kstD* and *hsaC* for cholesterol degradation and survival of the bacilli in macrophages (29, 33, 43) strongly supports the hypothesis that cholesterol degradation is essential for the survival of tubercle bacilli during infection.

Based on the present and previous findings, we hypothesize that during the early stages of infection, pathogenic mycobacteria bind and accumulate host cholesterol to buffer their internalization into macrophages and inhibit phagosome maturation. This accumulation might change cell signaling by affecting lipid rafts and may protect tubercle bacilli against toxins by decreasing their cell wall permeability. Thus, when other nutrients may be less available, cholesterol can become the source of carbon and energy, thereby allowing tubercle bacilli to survive long-term in the host.

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