Cholesterol is accumulated by mycobacteria but its degradation is limited to non-pathogenic fast-growing mycobacteria

Yossef Av-Gay and Rafat Sobouti

Abstract: In this report we show that fast-growing non-pathogenic mycobacteria degrade cholesterol from liquid media, and are able to grow on cholesterol as a sole carbon source. In contrast, slow-growing mycobacteria, including pathogenic *Mycobacterium tuberculosis* and bacillus Calmette-Guérin (BCG), do not degrade and use cholesterol as a carbon source. Nevertheless, pathogenic mycobacteria are able to uptake, modify, and accumulate cholesterol from liquid growth media, and form a zone of clearance around a colony when plated on solid media containing cholesterol. These data suggest that cholesterol may have a role in mycobacterial infection other than its use as carbon source.

Key words: mycobacteria, cholesterol, biodegradation.

Résumé : Notre étude confirme que les mycobactéries non pathogènes à croissance rapide sont capables de dégrader le cholestérol present dans les milieux liquides et d'utiliser le cholestérol comme seule source de carbone. À l'inverse, les mycobactéries à croissance lente, incluant *Mycobacterium tuberculosis* pathogène et le bacille de Calmette-Guérin (BCG), sont incapables de dégrader ou d'utiliser le cholestérol comme source de carbone. Les mycobactéries pathogènes sont néanmoins capables de capter, modifier et emmagasiner le cholestérol présent dans un milieu de culture liquide et à partir de la zone entourant les colonies qui se développent sur un milieu solide contenant du cholestérol. Les résultats obtenus suggèrent que le cholestérol aurait un rôle dans l'infection à mycobactéries autre que de servir de source de carbone.

Mots clés : mycobactéries, cholestérol, biodégradation.

[Traduit par la Rédaction]

Introduction

Mycobacterium tuberculosis is the leading bacterial cause of human mortality in the world. One-third of the world's population is considered to be exposed to this pathogenic organism, and each year it kills nearly three million people (Raviglione et al. 1995). The introduction of antibiotics and chemotherapy, together with an improvement in public hygiene, resulted in a dramatic decrease in mortality due to tuberculosis throughout the world. However, during the past decade there has been a dangerous re-emergence of tuberculosis in both industrialized and non industrialized nations, often involving multiple antibiotic-resistant strains. This clearly emphasizes the urgent need for effective new treatments.

The pathogenesis of any bacterial disease is a complex biological phenomenon involving many biochemical, genetic, and physiological aspects of both the host and the bacterial invader (Finlay and Falkow 1997). *Mycobacterium tuberculosis* is a facultative intracellular organism and its entry, its subsequent replication, and its ability to avoid destruction in the macrophage, are critical to its virulence. Since *M. tuberculosis* is capable of residing within host cells for a long period of time, it must have mechanisms to overcome nutrient limitations. There have been limited studies aimed at identifying carbon sources that are available for *M. tuberculosis* growth inside the macrophage. Mycobacteria, like most actinomycetes, can grow in vitro on an extremely wide range of carbohydrates and hydrocarbons. These oxidized carbon sources include alkanes, alcohols, ketones, and many mono-, di-, and tri-carboxylic acids (Ratledge 1982). With the exception of several glycolytic enzymes, the hexose monophosphate shunt, the Entner–Doudoroff pathway, and phosphoglucose isomerase, carbon metabolism in mycobacteria has not been well characterized. Glycerol is considered to be the preferred carbon source when mycobacteria are grown in vitro, although its utilization is blocked until all other carbon sources, including amino acids, have been consumed (Winder and Brennan 1966).

Cholesterol is the major sterol constituent of eukaryotic organisms, involved in stabilization of membranes as well as being a factor in hormonal and other signaling pathways (Lamb et al. 1998). With the exception of the cell-wall-deficient bacteria, *Mycoplasma*, the majority of prokaryotic organisms do not contain sterols in their membranes, and

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hapanoid is believed to fulfill the role of sterol in higher organisms (Taylor 1984). Nevertheless, cholesterol can be metabolized by a wide range of microorganisms as a carbon and energy source (Schatz et al. 1949; Fukuda et al. 1973; Li and Beitz 1996). The ability of actinomycetes, specifically mycobacteria, to bioconvert sterol is well documented, and has been utilized by the pharmaceutical industry to synthesize novel steroid compounds (Schoemer and Martin 1980). In human macrophages, cholesterol is available as an integral part of the cell membrane, including the phagosomal membrane. Since mycobacteria reside in macrophages, an important issue is whether pathogenic mycobacteria can utilize the cholesterol located in the phagosomal membrane that surrounds the tubercle bacilli. Although it has been proposed that pathogenic mycobacteria use lecithin and not cholesterol (Kondo and Kanai 1976*b*) in vivo as carbon source, a few reports indicate that cholesterol might be consumed by pathogenic mycobacteria. In addition, it has been shown that cholesterol is required for growth of pathogenic mycobacteria such as *M. scrofulaceum*, isolated from leprous tissues (Buki et al. 1969; Kato 1979). Interestingly, the complete genomic sequence of *M. tuberculosis* suggests that it contains sterol biosynthetic enzymes as well as two putative cholesterol degradation enzymes (Cole et al. 1998; Bellamine et al. 1998). Supportive evidence for the ability of mycobacteria to synthesize cholesterol appeared in a recent report which demonstrates that *M. smegmatis* is able to synthesize trace amounts of cholesterol (Lamb et al. 1998). Nevertheless, the physiological role of cholesterol and the enzymes involved in its biodegradation by bacteria, and specifically mycobacteria, remain unknown. This study examines and compares the ability of various mycobacteria to take up and utilize cholesterol supplied in their growth media in an attempt to determine whether mycobacteria use cholesterol as a carbon source.

Materials and methods

Strains, growth media, and sample preparation

Cultures of *M. smegmatis* MC^2155 (a kind gift of William Jacobs), *M. fortuitum* (ATCC 6841), and *M. phlei* (ATCC 354) were grown in Modified Dubos Medium (MDM). *Mycobacterium tuberculosis* H37*Rv* (ATCC 27294), *M. tuberculosis* H37*Ra (*ATCC 25177), *M. avium* (ATCC 25291), *M. intracellulare* (ATCC 13950), and *M. bovis* BCG strain Pasteur (ATCC 35734) were grown in MDM media, or in minimal medium containing the inorganic components of Proskauer-Beck medium without any glycerol as a carbon source. In each case, cholesterol (Sigma) was added to the media when required. The cholesterol was suspended in 5 mL Tween-80, heated briefly, and added to the media (1 g/L). For plate preparation, cholesterol was either suspended in 10 mL of 95% ethanol or in 20 mL of warm Tween-80. The solution was filtered through a Millex 0.22 μm filter and added to the media. Plates were made by adding pure agarose (FMC) to a final concentration of 15% as a solidifying agent. After growth and five-fold expansion over two weeks, cells were harvested by centrifugation for five minutes at 3750 × *g*. Cell culture media was filtered twice with 0.22-μm filter units (Nunc) and stored at –70°C until assayed. Cell pellets were washed once in lysis buffer (20 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.2 mM PMSF, 1 μg/mL pepstatin A, 1 μg/mL aprotonin, 0.5 mM sodium vanadate, 0.2 mM sodium molybdate), suspended in the same buffer, then subjected to mechanical bead breakage (Mini-Beadbeater, Biospec Products) for five minutes using 100 μm zirconia beads (Sigma) at room temperature. Cell debris was removed by centrifugation at 10 000 × *g* for 5 min. The resulting supernatant was filter-sterilized (Millex 0.22 μm, Millipore) and stored in aliquots at –70°C.

Cholesterol assay

Cholesterol levels were determined colorimetrically using a commercially available kit (Boehringer Manneheim Cat. No. 139050).

Thin layer chromatography

Thin layer chromatography of sterols was performed according to Stahl (Stahl 1969). Briefly, lipids, sterols, and sterol derivatives were extracted with a solution of chloroform-methanol 1:1 (v/v). A 40-μL volume of each phase was applied to silica gel thin layer chromatography (TLC) plates (Whatman 25) and separated using cyclohexane/chloroform 1:1 (v/v). Development and detection were performed by spraying with o-phosphoric acid/H_2O, 1:1 (v/v), followed by baking at 120°C for 15 min and spraying with freshly prepared 50 mg/mL phosphomolibdic acid ethanolic solution. Spots were stabilized after 5 min by heating plates at 85°C.

HPLC analysis

A 14 mL volume of media was passed through silica columns (abeselut NEXUS sorbent-Varian), rinsed with 1 mL H_2O, followed by vacuum drying. Hydrophobic compounds were eluted with 1 mL methanol. HPLC analysis was performed on Bondapak C-18 column (Waters). A 100-μL volume of the eluate from sorbent column was injected, and an isocratic acetonitrile/isopropanol 1:1 (v/v) wash was used to separate sterols. Cholesterol was detected at 210 nm.

Results

Growth on cholesterol as a sole carbon source is a property of non-pathogenic mycobacteria

To test whether mycobacteria are able to grow on cholesterol, a number of slow- and fast-growing mycobacterial strains were plated on minimal media containing cholesterol as a sole carbon source; all fast-growing mycobacterial strains tested were able to grow on cholesterol plates. As illustrated in Fig. 1A, *M. smegmatis* was able to grow on minimal medium containing cholesterol as a sole carbon source. The same was observed for *M. phlei* and *M. fortuitum* (data not shown). In contrast, slow-growing pathogenic-mycobacteria, such as *M. tuberculosis*, grew on this medium only when Tween-80 was used as the organic solvent (which by itself can be used as a carbon source). When left to grow for six weeks on solid media, slow-growing pathogenic mycobacteria such as *M. avium*, *M. intracellulare*, and two strains of *M. tuberculosis* (*Ra* and *Rv*) cleared cholesterol from the plates (Fig. 1B), while fast growers such as *M. fortiutum* and *M. smegmatis* did not. The clearing of cholesterol was demonstrated by the creation of a clear zone around the colony.

Cholesterol contribution to mycobacterial growth in liquid media

As seen in Fig. 2, growth analysis of both *M. smegmatis* and *M. phlei* demonstrates that cholesterol enhances their growth. Both *M. smegmatis* and *M. phlei* reached stationary phase after 48 h of growth in media containing cholesterol, while in minimal media, at least 96 h were needed to reach stationary phase. Furthermore, in the presence of cholesterol, the stationary phase of both cultures lasts longer when com-

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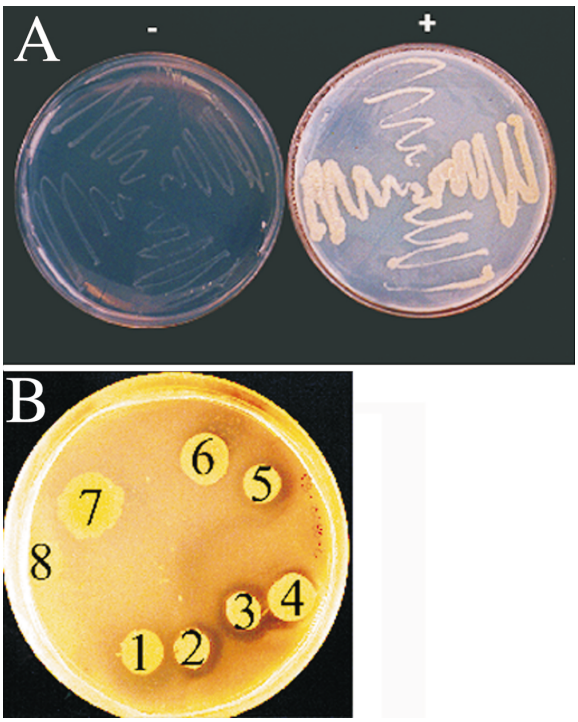
Fig. 1. (A) Growth of mycobacteria on cholesterol as a sole carbon source. A defined agarose based minimal medium without (–) or with (+) cholesterol as a carbon source was used to grow *M. smegmatis* for 48 h at 30°C. (B) Growth of mycobacteria on Tween-cholesterol plates. Slow growers: *M. tuberculosis* (2 and 3), *M. intracellulare* (1 and 6) and *M. avium* (4 and 5). Fast growers: *M. fortuitum* (7) and *M. smegmatis* (8).



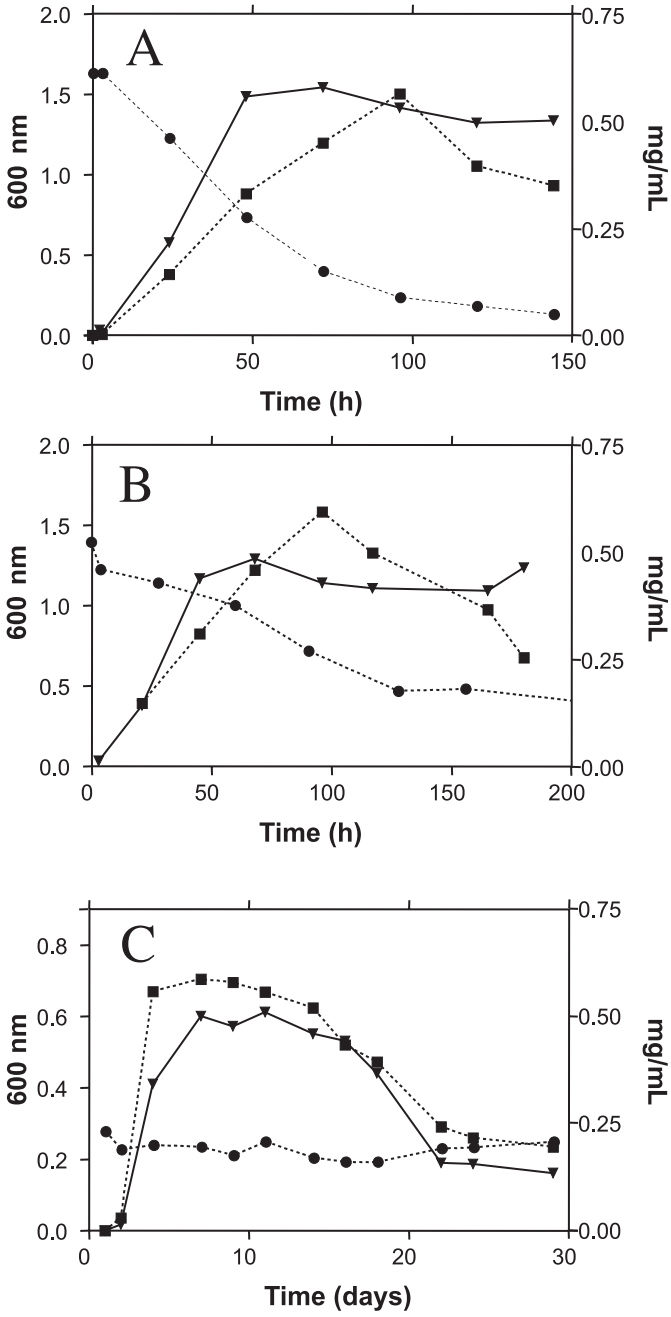
pared to the minimal media growth curve. On the other hand, when *M. bovis* BCG was examined as a representative of the slow-growing pathogenic mycobacteria, BCG growing in media containing cholesterol grew slower than that grown in minimal media lacking cholesterol (Fig. 2C). Furthermore, we noticed that higher levels of cholesterol in the growth media inhibited BCG growth.

Cholesterol accumulation and degradation by mycobacteria

Three different experimental approaches were used to determine cholesterol utilization by mycobacteria, specifically colorimetric determination of cholesterol levels, TLC, and HPLC analysis. Examination of cholesterol utilization during mycobacterial growth using the coupled cholesterol-oxidase and catalase colorimetric assay showed that *M. phlei* and *M. smegmatis* consumed cholesterol from the growth media throughout their growth cycle (Fig. 2A and 2B). Examination of cholesterol utilization by BCG during its growth curve (Fig. 2C) showed clearly that BCG did not remove cholesterol from its growth medium.

The use of a second approach, TLC analysis of cholesterol levels, was used in order to rule out the possibility that mycobacteria accumulate cholesterol from the growth media rather than degrade it, and to determine whether the small decrease in cholesterol levels during the first two days of BCG growth can be explained by its accumulation-deposition into the mycobacterial cell wall. As seen in Fig. 3,

Fig. 2. Growth curve and cholesterol utilization by (A) *M. smegmatis*, (B) *M. phlei*, and (C) BCG in liquid media. Results are representative of three independent experiments. Solid squares represent growth (A 600 nm) on MDM media and solid triangles are growth in the presence of 0.5 mg/mL cholesterol in MDM media for the fast growers and 0.25 mg/mL for BCG. Solid circles represent amount of cholesterol in the media. Initial cholesterol levels were titrated to give optimal growth of each strain.



M. smegmatis (A) and *M. phlei* (B) growth media and cell-free-extracts TLC showed (*i*) initial accumulation of cholesterol inside cells, and (*ii*) that cholesterol is disappearing

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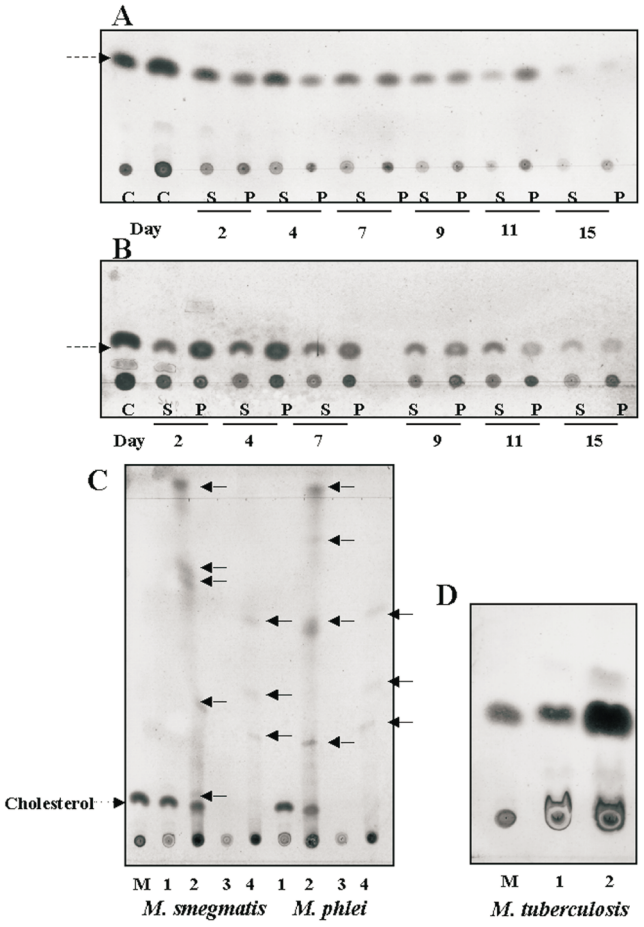
Fig. 3. Cholesterol degradation in mycobacteria. TLC analysis of cholesterol utilization by *(A) M. smegmatis* and (B) *M. phlei* during their growth curve. Culture volumes of 1.5 mL were fractionated by centrifugation to supernatant (S) and cells (P) before applying to TLC. (C) TLC analysis of sterols, marked by arrows, in *M. smegmatis* and *M. phlei*. **M**, control media + cholesterol. **1**, Growth media from 16-day-old culture containing cholesterol; **2,** cell-free extract from 16-day-old culture grown in the presence of cholesterol; **3**, growth media from 16-day-old culture without cholesterol; **4**, cell-free extract from 16-day-old culture grown without cholesterol. (D) TLC analysis of sterols in *M. tuberculosis* H37*Rv*. **M**, Media containing cholesterol; **1**, media from six-month-old culture grown in the presence of cholesterol; **2**, cell-free extract from six-month-old culture grown in the presence of cholesterol. No spots were observed when cholesterol was omitted from the growth media.



from both growth media and the cell extract. Further quantitative analysis of media and cell samples from late stationary phase of *M. phlei* and *M. smegmatis* (Fig. 3C), grown in the presence or absence of cholesterol, showed clearly that cholesterol is degraded. Furthermore, cholesterol degradation sterol by-products can be detected in cultures grown in the presence of cholesterol, but not in medium from cells that grew without cholesterol. On the other hand, TLC analysis of *M. tuberculosis* H37*Rv* and BCG growth media, and cell free extracts, showed that cholesterol is present in the growth media, and that there is an accumulation of cholesterol within *M. tuberculosis* cells (Fig. 3D). Cholesterol did not disappear from the growth media even when the culture was left to grow for over six months. Identical results were obtained with cultures of BCG. Furthermore, using a radioactive assay, we were not able to identify degradation products of cholesterol in *M. tuberculosis*, *M. avium*, or BCG cultures (data not shown).

HPLC analysis is probably the most sensitive method for the detection of trace amounts of sterols. As a confirmation to the above results, analysis of late stationary phase growth media, (Fig. 4), showed clearly that cholesterol is completely depleted from *M. smegmatis* and *M. phlei* culture media, while it can be detected in *M. bovis* BCG culture media even after approximately four weeks of growth.

Discussion

In this report, we demonstrate that fast-growing nonpathogenic mycobacteria are able to degrade and utilize cholesterol when it is supplied as a sole carbon source. On the other hand, slow-growing mycobacteria, including those that are pathogenic, utilize cholesterol in a different manner. We have shown that *M. tuberculosis* and BCG accumulate cholesterol, but do not seem to metabolize it. The ability to degrade cholesterol has been shown for several species including *Streptomyces*, *Brevibacterium*, and several nonpathogenic fast-growing mycobacteria (Schoemer and Martin 1980). Biochemical descriptions of cholesterol degradative enzymes, such as cholesterol oxidase, exist for a wide variety of microorganisms including Gram-negative enterococci (Li and Beitz 1996) and Gram-positive bacteria (Solaiman and Somkuti 1991). In mammals, there is no complete degradation of cholesterol. Cholesterol is modified by either 7-α-hydroxylation to form bile acids, or by oxidation of the 3-β-hydroxyl group with additional hydroxylation and oxidation of the side chain to form cholic acid or steroid hormones. In these cells, steroids are hydrolysed by mixed function oxidases that utilize NADPH and O_2. All of these reactions involve cytochrome P450. In contrast to mammals, bacteria are able to degrade cholesterol, with CO_2 and H_2O as the final products. Two different bacterial pathways for cholesterol degradation are represented by two different families of the cholesterol oxidase enzymes (Wilmanska et al. 1995). Fast-growing mycobacteria and *Rhodococcus* cholesterol oxidases have a cascade of enzymes that take part in steroid biodegradation, while the producers of extracellular cholesterol oxidases such as *Streptomyces* spp., and *Arthrobacter* were suggested to be incapable of decomposing sterols (Wilmanska et al. 1995). The above classification is in agreement with our results which show clearly that fast-growing mycobacteria degrade cholesterol, but do not clear it from their growth media. Our demonstration that slow-growing pathogenic mycobacteria clear cholesterol from their growth media, probably via secretion of the enzyme cholesterol oxidase, is further support for the hypothesis that cholesterol oxidases are different in organisms that fail to degrade cholesterol after its uptake.

By ruling out the nutritional role of cholesterol for pathogenic mycobacteria, one needs to provide reasons for the observation that cholesterol is accumulated in the cells. A few hypotheses may be considered. Cholesterol has been shown to play a regulatory role in several systems by modifying

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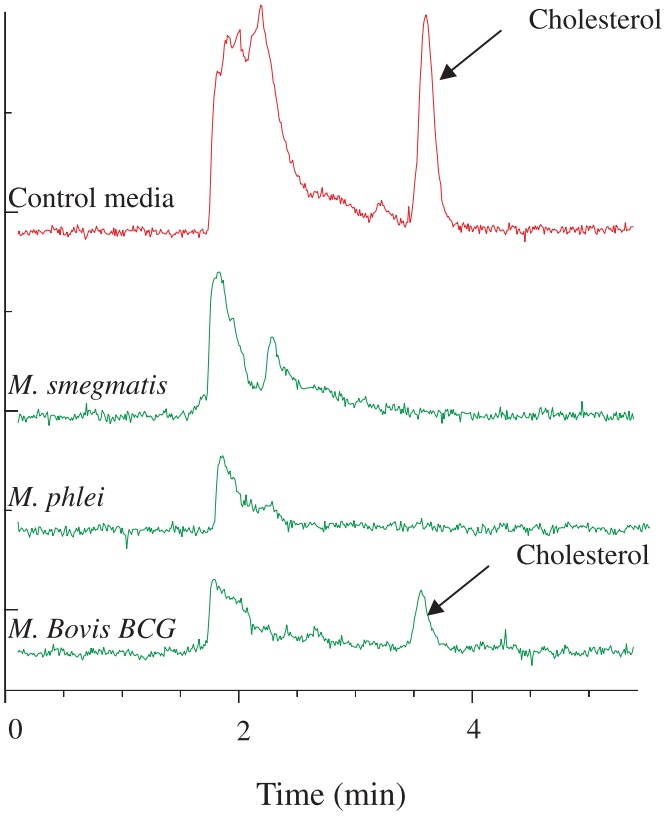
Fig. 4. HPLC analysis of mycobacterial growth media. Growth media from late stationary phase *M. smegmatis* (19 days), *M. phlei* (19 days), and *M. bovis* BCG (29 days) cultures were separated on C-18 reverse phase column as described in Materials and methods.



signaling proteins, and thus cholesterol accumulation and oxidation by intracellular mycobacteria may effect host cell signaling (Porter et al. 1996). The change in the membrane balance or cholesterol solubility by its oxidation may lead to the prevention of phagosomal maturation. The third possible function of cholesterol oxidation is that mycobacteria may import the soluble cholesterol into their cell wall surface. If this hypothesis is true, then this provides mycobacteria with another mechanism for "hiding" inside macrophages by mimicking eukaryotic membranes. Furthermore, incorporated cholesterol may act as free-radical- and toxic-metabolite scavengers. In support of the last two hypotheses, free cholesterol, its esters, and phthiocerol dimycocerosate (DIM) constituted the major portion of neutral lipid extracts from *M. bovis* grown in vivo (Kondo and Kanai 1976*a*). Cholesterol and cholesterol ester levels in macrophages infected with mycobacteria have been shown to be much higher when compared with non-infected cells both in vivo and in vitro (Kondo and Kanai 1976*a*, 1976*b*, 1977). Related studies show that coating mycobacteria with cholesteryl oleate, with or without DIM, had an infection-promoting effect resulting in longer persistence of avirulent bacilli, and less sensitivity to the protective mechanisms generated by BCG immunized mice against virulent tubercule bacilli (Kondo and Kanai 1976*a*). Current activities in our laboratory will explore the role of cholesterol in *M. tuberculosis* growth and pathogenisis. The cholesterol oxidase enzyme has been cloned and expressed in *E. coli* (Av-Gay et al. 1997), and gene knockout experiments to reveal its role in *M. tuberculosis* growth and pathogenisis are underway.

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