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In Vitro Cultivation of Mycobacteria in Cholesterol Lecithin Media from Lepromas of Rats Infected with *Mycobacterium lepraemurium* [1]

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The growth requirements for mycobacteria are fairly simple ([38]). However, in order to obtain growth with only small numbers of bacilli in the inoculum from cultures or from clinical or other specimens it is necessary to incorporate into the medium such biologic materials as animal serum, serum fractions, egg yolk or starch. Even in these conditions many strains of tubercle bacilli grow only in weeks or months and several strains are still noncultivable in artificial media. Specific substrates and nutrients were necessary to isolate cultures of hitherto not fully characterized strains of mycobacteria from human and rat leprous tissues. The isolation cultures soon became adapted to conventional culture media in which cultivable mycobacteria multiply ([15,16]). Ogawa ([25]) claimed the cultivation of *M. lepraemurium* on egg yolk medium and this extremely slow growing strain still does not grow on any of the known semisolid or liquid culture media except egg yolk ([22,24,25]).

The use of complex biologic materials in media has several disadvantages. The chemical constitution of egg yolk varies with breed and diet. Animal sera differ in composition and are subject to great daily variations depending on diet, age, climate and health conditions of the donor animal. Serum albumin fractions are not easily reproducible. The preparation of the media containing egg yolk, serum or serum fractions requires special equipment for inspissation, sterilization by filtration and storage. These media are expensive, troublesome to prepare, not easily reproducible, and, thus, not entirely reliable.

Several attempts have been made to overcome these drawbacks ([4,5,9,10]), however most of the media currently in use for the isolation of *M. tuberculosis* contain egg yolk, serum or serum fractions. Searching for media for the cultivation of *M. leprae* and *M. lepraemurium*, Kato ([12,13]) reported that cholesterol was necessary for *in vitro* growth of mycobacteria from leprous tissues from armadillos (*Dasypus novemcinctus* L.) infected with *M. leprae*. We are now able to report that hitherto non-identified strains of mycobacteria can be grown regularly from lepromas of rats infected with *M. lepraemurium*. We also confirm the findings of Ogawa ([25]) and Koseki *et al* ([22]) that strains of mycobacteria can be cultivated on the Ogawa egg yolk medium inoculated with *M. lepraemurium* from rat leprous tissues. Further, we will here describe that the culture claimed by Ogawa as *in vitro* grown *M. lepraemurium* can be grown in the liquid cholesterol-lecithin medium described by Kato ([12,13]).

MATERIALS AND METHODS

Microorganisms. *In vivo* grown *M. lepraemurium* was isolated from a four month old subcutaneous rat leproma, Hawaiian strain, maintained for 25 years in Sprague-Dawley rats. Preparation of *in vivo* grown *M. lepraemurium* suspensions has been described elsewhere ([17]). *In vitro* grown *M. lepraemurium*, the Hawaiian strain utilized by Ogawa, was received from K. Kohsaka, Department of Microbiology, Osaka University. The strain has been transferred at six week intervals on Ogawa egg yolk medium ([25]).

Culture media. The phosphate buffer solution contained 8.2 gm KH_2PO_4 and 0.5 gm Na_2HPO_4 in one liter distilled water. After

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sterilization in an autoclave for 25 minutes at 15 lbs, the buffer was of pH 5.8. The basal medium contained 8.2 gm KH_2PO_4, 0.5 gm Na_2HPO_4, 4 gm yeast extract (Difco) and 30 ml glycerol in one liter distilled water.

Cholesterol-lecithin medium was prepared as described by Kato [12]. Two hundred milligrams cholesterol, reagent grade, were dissolved in 4 ml warm acetone. This solution was injected with a needle attached to a syringe into one liter of basal medium. The medium was autoclaved for ten minutes to evaporate the acetone. Two hundred milligrams of L-α lecithin reagent grade were carefully homogenized in 20 ml phosphate buffer solution. This solution was injected with a needle attached to a syringe into the cholesterol-basal medium cooled to room temperature. Nine milliliters of the medium were distributed into each of 50 ml screw cap tubes. The media were sterilized for 25 minutes at 15 lbs in an autoclave. One milliliter of filter sterilized sheep serum was added aseptically to each of the tubes containing 9 ml cholesterol-lecithin basal medium. Reaction of the medium was pH 5.8 to pH 6.1.

Ogawa egg yolk medium [25] was used. One gram KH_2PO_4 and 1 gm Na glutamate were dissolved in 100 ml distilled water with 6 ml glycerol added. The solution was sterilized in an autoclave for 20 minutes. Two hundred milliliters of egg yolk were added to the solution and mixed in a Waring blendor under aseptic conditions. The medium was distributed in sterile 50 ml screw cap tubes in approximately 10 ml amounts. The medium was coagulated in a slanting position at 90°C for 50 minutes. Reaction of the medium was pH 5.9 to pH 6.1.

Boiled egg yolk filtrate medium. One egg yolk was homogenized with a Waring blendor in 80 ml distilled water and 10 mg crude trypsin (pancreatic type II). The homogenate was digested for 60 minutes at 37°C with occasional shaking after which 900 ml of basal medium were added to the trypsin digested egg yolk homogenate, mixed and autoclaved for 15 minutes at 15 lbs. The medium was filtered through standard filter paper while hot and the precipitate was washed through the same filter paper with adequate amounts of basal medium to obtain one liter of filtrate. After cooling to room temperature, the filtrate was again filtered through filter paper. The filtrate was distributed and autoclaved in the same way that was used for the cholesterol-lecithin medium. Sheep serum, 10% v/v was added aseptically to the medium when cooled to room temperature.

Semisolid media were prepared by adding 1.5% agar (Difco) to the liquid medium. Following sterilization in an autoclave for 30 minutes the agar slants were prepared with or without aseptically added serum.

Inoculation of liquid media and estimation of growth ([16]). Prior to inoculation, 200 units per ml of sodium penicillin G were added to the media. The inoculated media were incubated at 34°C. The carefully homogenized suspensions of mycobacteria isolated from the rat lepromas or bacilli from the *in vitro* grown cultures were inoculated into the liquid culture media under aseptic conditions. The inoculum was suspended and diluted in phosphate buffer so that the inoculated liquid media contained a relatively high number of acid-fast bacilli. The concentration of the acid-fast bacilli in the media was determined as a relative base line eight to twelve hours after inoculation, estimating the relative density of the inoculum in the liquid media. For this purpose the culture was vibrated on a shaker for five to ten seconds to achieve homogenization of the inoculum in the medium. A loopful of the inoculated medium, using a 2 mm platinum loop, was placed on a slide and spread evenly over a 1 cm diameter surface. The preparation was dried in a paraffin oven at 60°C and fixed by flaming. Since young cultures of such mycobacteria are usually non-acid-fast, easily decolorized and often not detected with conventional technics, the preparations were treated with periodic acid solution to reveal those forms of mycobacteria which would otherwise remain undetected. A 5–10% aqueous solution of periodic acid was poured over the slide and heated by flame until the first signs of evaporation became visible. Two minutes later the preparations were washed with tap water and stained by the Ziehl-Neelsen method. The number of bacilli per microscopic field, scanning diagonally on the 1 cm diameter preparation, was determined. The average number per field was set as a base line for evaluation of cultivation progress.

The base line was usually 50 to 75 bacilli per field depending on the size of the inoculum. The progress of cultivation was estimated by the appearance of a sediment or turbidity in the liquid medium and microscopically by the increase in the number of bacilli in the stained preparations. Again, for practical purposes, the progress of cultivation was estimated and graded as — (no growth) to ++++ (heavy growth) as described previously ([16]).

Inoculation of semisolid media and estimation of growth. Ogawa egg yolk media, the semisolid cholesterol-lecithin and semisolid boiled egg yolk media were inoculated with extremely heavy sediments of the centrifuged purified suspensions of host-grown *M. lepraemurium* or with *in vitro* grown cultures. A loopful of the packed sediment was placed gently on the surface of the semisolid slants of media. The inoculum was spread with the loop over a 1 to 2 cm^2 area. When cultivated bacilli were transferred to semisolid media a similar procedure was used or a surface grown colony of bacilli was transferred from semisolid to semisolid media. This heavy inoculum or transfer was necessary to obtain isolation cultures or subcultures as pointed out by Ogawa ([25]) and by the authors ([22, 24]) confirming the results of Ogawa. Growth was estimated on the surface of the semisolid media by the appearance of constant growth of colonies, confirmed by microscopic examination of the bacilli which formed the colonies.

Measurements of oxidation of substrates. Conventional Warburg manometric technics were employed ([32]). Experiments were conducted at 34°C with air as the gas phase. Each Warburg flask contained 10 mM of substrate in 0.1 M potassium phosphate buffer, pH 5.6 in the side arm and 1.0 ml of cell suspension in the main compartment. All cell suspensions were standardized photometrically. The homogenized bacilli were diluted with 0.1 M potassium phosphate buffer, pH 5.6, so that a 1:10 dilution of the cell suspension gave 300 Klett units at 540 nM which corresponded to 10 mg dry weight of cells in 1 ml of final cell suspension. When yeast extract was used as a substrate, 0.2 ml of a 10% solution was used. CO_2 was absorbed by 0.2 ml of 20% KOH in the center well. The total volume of liquid in the Warburg flasks was 2.0 ml. Additional control in all experiments consisted of simultaneous determination of endogenous respiration. The flasks were equilibrated for 15 minutes prior to the addition of the substrate from the side arm.

Cholesterol in the in vivo and in vitro grown bacilli. Packed whole bacilli were obtained by centrifugation at 12,000 × g for 20 minutes of the purified host-grown *M. lepraemurium* cell suspensions and of the *in vitro* grown strain of mycobacteria from the same rat for the determination of total cholesterol content in whole cells.

Cell wall, bacterial membrane fragments and soluble extract were prepared from *in vivo* and *in vitro* grown cell suspensions. Purified cell suspensions were passed four times through a chilled French Pressure Cell at 2,000 psi. A crude, cell-free extract was obtained by centrifugation of the homogenate at 20,000 × g for 30 minutes. The sediment was resuspended in 0.1 M potassium phosphate buffer, pH 5.8, and again passed four times through the French Pressure Cell to obtain cell walls free of unbroken cells. The resulting homogenate was centrifuged at 20,000 × g for 30 minutes. The sediment was washed twice with PO_4 buffer to obtain cell walls free of cell-free extract for cholesterol estimation. To obtain particles or membrane fragments, crude cell-free extract was centrifuged at 143,000 × g for 90 minutes in a Beckman L-G5 ultracentrifuge. The resulting particulate fraction which contained cell membranes was used for cholesterol determination.

Determinations of total cholesterol were made utilizing separately 0.5 gm (wet weight) each of the lepromas, purified bacilli or in the cell walls, or the cell membranes prepared from 0.5 gm (wet weight) of bacilli. Extraction of cholesterol from each specimen was accomplished by the following method. Each fraction weighing 0.5 gm was homogenized thoroughly with 5 ml 1:1 acetone-methanol mixture (v/v) in a glass homogenizer and centrifuged at 4,000 rpm for five minutes. The clear extract was removed and the sediment was extracted three more times with the same solvent. The extracts were pooled in a test tube and evaporated by placing the tube in cold water and bringing the water to boiling. Five milliliters of glacial acetic acid were added to each fraction and the total cholesterol determined

TABLE 1. *Oxidation of various substrates by* in vivo *grown* M. lepraemurium *isolated from rat lepromas.*

Substrates	Exogenous O^2 uptake μ½ hours
Glycerol 10 mM	0
Na glutamate 10 mM	0
Cholesterol 10 mM	0
Lecithin 10 mM	0
Egg yolk 20 mg	0
Boiled filtrate of 20 mg egg yolk	0
Yeast extract (Difco) 20 mg	46

TABLE 2. *Growth of mycobacteria in media inoculated with* M. lepraemurium *isolated from lepromas of rats (growth was estimated and graded from — [no growth] to ++++ [heavy growth]).*

Media	Estimated growth at 34° C Days: 20	30	40
PO_4 buffer pH 5.8	—	—	—
Same plus serum	—	—	—
Cholesterol-lecithin, yeast extract	—	—	—
Same plus serum	—	—	—
Cholesterol-lecithin, yeast extract, glycerol	—	—	—
Same plus serum	++	+++	++++
Cholesterol-lecithin, yeast extract, glycerol, agar	—	—	—
Same plus serum	—	—	—
Ogawa egg yolk	—	±	++
Same plus yeast extract	+	++	++++
Boiled egg yolk filtrate, glycerol	—	—	—
Same plus serum	—	—	—
Boiled egg yolk filtrate, glycerol, yeast extract	—	±	±
Same plus serum	++	+++	++++
Same plus agar and serum	—	—	—
Lowenstein-Jensen	—	—	—
Dubos oleic acid, serum albumin liquid	—	—	—
Sauton liquid	—	—	—

by the method of Wycoff and Parsons ([37]).

Oxidation of substrates of the culture media. Glycerol, Na glutamate, cholesterol, lecithin, egg yolk or boiled egg yolk filtrate were not oxidized in the Warburg apparatus by host-grown *M. lepraemurium* suspensions isolated from rat lepromas. Yeast extract was oxidized at a low rate, but regularly with 22 different batches of host-grown *M. lepraemurium.* Results are shown in Table 1.

Growth of mycobacteria in the media inoculated with host-grown M. lepraemurium (Table 2). No multiplication of bacilli occurred in the phosphate buffer solution with or without serum added during 40 days of incubation. No growth occurred in the absence of glycerol in the cholesterol-lecithin, yeast extract media with or without added serum. Bacilli multiplied, however, in the complete cholesterol-lecithin, yeast extract, glycerol medium but only if enriched with sheep serum. Growth occurred within five to ten days and progressed to a heavy growth in 40 days. With 1.5% agar in the same media no growth whatsoever was detected on the surface of the semisolid slants during 40 days of observation. Growth was visible as an easily suspended sediment in the liquid media. Microscopically the bacilli were short and acid-fast only after periodic acid oxidation during the first one to two weeks of cultivation. With growth progressing, bacilli formed clumps of different sizes and became strongly acid-fast.

An incipient growth was observed on the surface of the Ogawa egg yolk medium after 30 days of incubation. At 40 days the growth became more visible as slowly growing, round, slightly yellowish-grey colonies. However, on the egg yolk media enriched with yeast extract visible growth was observed in 20 days, progressing to a heavier growth in the following three weeks, but thereafter no further growth occurred. The visible morphology of the colonies was the same as on egg yolk slants without yeast extract. In stained preparations bacilli were long and strongly acid-fast with a tendency to agglomerate on egg yolk media with or without yeast extract. Yeast extract had a definite enhancing effect on the growth of bacilli on the Ogawa egg yolk medium.

In the boiled egg yolk filtrate media growth occurred only in the presence of glycerol, yeast extract and serum. However, the growth was observed only in the liquid media. The growth rate was comparable with multiplication in the cholesterol-lecithin, glycerol, yeast extract, serum medium. In

TABLE 3. *Growth of mycobacteria in media inoculated with strains of mycobacteria cultivated from rat lepromas.*

I. Cholesterol-lecithin, yeast extract, glycerol, serum (2nd subculture).
II. Ogawa's egg yolk medium, Montreal strain (2nd subculture).
III. Ogawa's egg yolk medium, Ogawa strain (numerous subcultures).

Media	Estimated growth at 34°C[a]					
	Strain I		Strain II		Strain III	
Days:	5	10	5	10	5	10
PO_4 buffer pH 5.8	—	—	—	—	—	—
Same plus serum	—	—	—	—	—	—
Cholesterol-lecithin, yeast extract	—	—	—	—	—	—
Same plus serum	—	—	—	—	—	—
Cholesterol-lecithin, yeast extract, glycerol	±	+	±	+	—	—
Same plus serum	++	++++	++	++++	+	+++
Cholesterol-lecithin, yeast extract, glycerol, agar	—	—	—	—	—	—
Same plus serum	—	—	—	—	—	—
Ogawa egg yolk	—	—	—	+	—	+
Same plus yeast extract	—	++	—	++	±	+
Boiled egg yolk filtrate, glycerol	—	—	—	—	—	—
Same plus serum	—	—	—	—	—	—
Boiled egg yolk filtrate, glycerol, yeast extract	—	—	—	—	—	—
Same plus serum	++	+++	++	++++	+	+++
Same plus agar and serum	—	—	—	—	—	—
Lowenstein-Jensen	—	—	—	—	—	—
Dubos oleic acid, serum albumin liquid	—	—	—	—	—	—
Sauton liquid	—	—	—	—	—	—

[a] Later subcultures of strain I were adapted to grow on Lowenstein-Jensen, but not on Dubos and Sauton liquid media. No adaption yet with strains II and III.

both liquid media growth characteristics and morphology of the bacilli were the same. Again no growth occurred on the slants of semisolid media with 1.5% agar. Bacilli did not grow on Loewenstein-Jensen or in Dubos oleic acid serum albumin or Sauton liquid media within two months of incubation.

Growth of the in vitro grown bacilli in subcultures (Table 3). The isolation culture of the strain of mycobacteria grown in the liquid cholesterol-lecithin, yeast extract, glycerol, serum medium was transferred to a media enumerated in Table 3. Again multiplication occurred only in the homologous media, in the enriched boiled egg yolk filtrate and on the Ogawa egg yolk medium with and without yeast extract added. While growth was slow and poor on the surface of the Ogawa egg yolk medium, it rapidly progressed on egg yolk slants enriched with yeast extract. The growth enhancing effect of yeast extract became evident and agar completely inhibited multiplication on the semisolid media.

The isolation culture obtained on the Ogawa egg yolk medium is designated as the "Montreal strain" in Table 3. This culture when transferred into subcultures grows only on the same media as the isolation culture obtained on cholesterol-lecithin, yeast extract, glycerol, serum medium. The culture is now regularly subcultured in the enriched complete cholesterol-lecithin liquid media. After several subcultures growth also developed on the agar slants of the same media.

The *in vitro* grown Hawaiian strain of *M. lepraemurium*, received and maintained on Ogawa egg yolk medium, is designated in Table 3 as the "Ogawa strain". This was successfully subcultured on the Ogawa egg yolk medium with enhanced growth when the medium was enriched with yeast extract. Heavy growth of this slow growing culture developed in the complete liquid cholesterol-lecithin media and in the complete boiled egg yolk filtrate. No growth occurred on the surface of the agar slants and on or in conventional media used for the cultivation of mycobacteria. Growth was better supported in the liquid enriched cholesterol-lecithin

media than on the Ogawa egg yolk slants. Growth characteristics of the Ogawa strain in the three media in which growth occurred was the same as with the Montreal strain.

Cholesterol and the in vivo and in vitro grown cells. Total cholesterol content of *in vivo* grown *M. lepraemurium* was 4.10 mg per gm wet weight of cells. Most of the cholesterol was concentrated in the cell membranes. In the cell-free extract, centrifuged for 90 minutes at 145,000 × g, the sediment obtained from 1 gm *M. lepraemurium* cells contained 3.20 mg cholesterol. Cell walls from 1 gm of cells containing 0.64 mg and 0.19 mg cholesterol was measured in the supernatant of the 145,000 × g centrifugate.

In the whole cells of the M.R56 strain cultivated *in vitro* from a rat leproma, the cholesterol content was 7.03 mg/gm wet cells when grown in the cholesterol-lecithin medium. Most of the cholesterol was concentrated in the cell membrane fraction. Results are shown in Table 4.

TABLE 4. *Total cholesterol content in subcutaneous rat leproma, purified cells of* Mycobacterium lepraemurium, *cell walls and cell membranes as well as mycobacteria cultivated from rat lepromas* (Mycobacterium *R56*).

Material	Total cholesterol mg/gm cells (wet)
Subcutaneous rat leproma, 4 months old	3.69
M. lepraemurium, purified cell suspension	4.10
M. lepraemurium, cell walls	0.64
M. lepraemurium, cell membranes (145,000 × g)	3.20
M. lepraemurium, cell-free extract supernatant (145,000 × g)	0.19
Mycobacterium R56 grown in yeast extract, cholesterol, lecithin, glycerol, serum	7.03
Mycobacterium R56 cell walls	0.82
Mycobacterium R56 cell membranes (145,000 × g)	5.08
Mycobacterium R56 cell-free extract supernatant (145,000 × g)	0.28

DISCUSSION

The pathologic manifestations of murine leprosy are characterized by an undisturbed host-parasite relationship. The causative agent, *M. lepraemurium*, multiplies slowly but abundantly in the host macrophages without any damage to the host cell which in turn is unable to destroy the murine leprosy bacilli multiplying in the phagocytic vacuole. *M. lepraemurium* is a nondigestible particle for the host macrophage. The substrates used for growth, multiplication and virulence by *M. lepraemurium* in the host macrophage are unknown. Since the macrophage is a rich reservoir of nearly any enzyme necessary for digestion of foreign matter and molecules, the cytoplasm of the macrophage is an excellent, and so far the only culture medium for *M. lepraemurium*, similar to *M. leprae*, but only in susceptible hosts. However, in the *in vitro* surviving macrophages, the multiplication of *M. leprae* and *M. lepraemurium* is either very limited or not satisfactorily proven. Macrophages must harbor a growth-promoting factor for both these hitherto noncultivable, but metabolically most competent strains of the genus of mycobacteria.

More and more data is accumulating which indicates that the macrophage has a dynamic storage capacity and enzymatic machinery for cholesterol metabolism ([19, 33-36]), and that the cells are a rich source of cholesterol for ingested mycobacteria which have a unique preference for accumulation ([3, 18-21, 29-31]) and esterification of cholesterol ([27]). Recently Kato ([13, 16]) proposed cholesterol as a growth factor for *in vitro* cultivation of mycobacteria in media inoculated with host-grown *M. leprae*. Now it seems that cholesterol has similar growth enhancing properties for mycobacteria from murine lepromas.

The intracellular fate of cholesterol and cholesterol esters in the macrophages was investigated by Werb ([33]) and Werb and Cohn ([34-36]). Macrophages actively exchange free cholesterol molecules with serum lipoproteins. Cholesterol is associated with the plasma membrane and with intracellular membranes. Cholesterol is localized within cytoplasmic phagolysosomes when dead erythrocytes, tissue cells, debris and chylomicrons are phagocytized by macrophages. Especially important is the finding by Werb and Cohn ([36]) that endocytosis of nondigestible substances results in the accumulation of cholesterol in the phagolyso-

somal membrane. There is a constant movement from membrane to membrane of the intracellular compartments and an exchange of cholesterol in and out of the macrophage. Cholesterol esterase hydrolyzes cholesterol within the secondary lysosomes of the macrophage. Thus, there is free cholesterol available to the ingested mycobacteria in the macrophage.

Mycoplasmas ([6,7]) and mycobacteria have a unique ability for cholesterol uptake and utilization. Tak ([30]) reported that earlier workers such as Söhngen in 1931 and Haag in 1933 cultivated mycobacteria in media with cholesterol as the sole source of carbon. Tak found that in several cultures of mycobacteria "cholesterol disappeared with relatively great speed" from media with cholesterol as the only source of carbon. Bleecken and Schubert ([3]) reported the "surprisingly fast utilization of cholesterol" by mycobacteria. Barksdale *et al* ([2]) cultivated L phase type organisms in a medium containing cholesterol and inoculated with host-grown *M. leprae*. Hirsch ([9,10]) found that cholesterol replaced egg yolk and serum in media for the cultivation of tubercle bacilli.

In our present experiments cholesterol can definitely be considered as a growth factor since it has not been possible to obtain growth of mycobacteria from rat lepromas, except in media containing added cholesterol or biologic material containing cholesterol. The same results were obtained by Kato ([12]) who cultivated mycobacteria from armadillos infected with *M. leprae*. Cholesterol is not a source of carbon for the strains since no growth was registered in media with cholesterol as the only source of carbon. Neither is cholesterol a source of energy since the sterol was not oxidized by the host-grown cells in the Warburg apparatus. It is, however, certain that host-grown *M. lepraemurium* and *in vitro* cultivated bacilli had a special affinity for cholesterol uptake from the medium. Cholesterol, however, did not replace cholesterol containing serum in our media since no growth occurred in the absence of serum. These results suggest that two growth promoting substances are probably necessary for the cultivation of the described strains, one being free cholesterol and another residing in the complexity of the serum. The mechanism by which cholesterol enhanced bacterial growth remains to be determined.

The *in vitro* cultivated *M. lepraemurium*, Ogawa strain, had unusual characteristics ([22]). The strain did not grow on any media except the Ogawa egg yolk medium. We are now able to grow the Ogawa strain in the cholesterol-lecithin, yeast extract, glycerol liquid medium enriched with sheep serum and on a liquid medium containing boiled egg yolk filtrate. The latter contained natural cholesterol in colloidal solution. This is the first time that the Ogawa strain of *M. lepraemurium* was successfully subcultured in liquid media. The growth in these liquid media was considerably faster and more abundant than in the egg yolk medium of Ogawa. Our studies in the Warburg apparatus showed that none of the ingredients of the Ogawa medium were oxidized by the host-grown *M. lepraemurium*. It is therefore possible that the Ogawa medium contains no substrate which *M. lepraemurium* can use for direct oxidative phosphorylation. The energy source in the Ogawa medium must therefore either be of small concentration or of low value, thus permitting an extremely slow growth of *M. lepraemurium*. In previous experiments we described yeast extract as an oxidizable substrate by host-grown *M. lepraemurium*. ([15]). When yeast extract is incorporated into the egg yolk medium the growth is considerably enhanced and the visible appearance and constant growth of the colonies is significantly accelerated.

It became evident in the experiments of Hirsch ([9]) that agar is toxic for tubercle bacilli. Our results clearly showed that 1.5% agar completely inhibited the growth of the three strains when added to the enriched cholesterol-lecithin or to the boiled egg yolk filtrate media. The primary cultures grow abundantly in the two liquid media, but not on the slants of agar containing semisolid media. The absence of growth on agar slants is certainly not due to differences in partial tension of O_2 since the bacilli multiplied well when exposed to O_2 on the surface of the Ogawa egg yolk medium.

Sakurai and Skinsnes ([26]) found an unusually high lipid content in host-grown *M. lepraemurium*. The free cholesterol content of the purified suspensions of the bacilli was 6.8 mg/gm wet weight of *M. lepraemurium* cells. These authors found that as high as 20% of the total lipid content of the skin lep-

roma in mice was free cholesterol. In our present experiments we similarly found high amounts of cholesterol in the host-grown and *in vitro* grown cells and the cholesterol content in the cell membranes in both cases. These findings indicate that cholesterol is not simply attached to the cell surface but integrated into a subcellular structure. In this respect the subcellular distribution of cholesterol in the host-grown *M. lepraemurium* as well as in the M.R56 strain shows a striking similarity to the localization of cholesterol in the macrophages ([33]) and in other mammalian cells. It is known that in mammalian cells cholesterol is a necessary building block for cellular integrity similar to the role of cellulose in the plants. Cholesterol might play a similar role in the cellular structure of *M. lepraemurium.*

Since cholesterol is insoluble in water, difficulties experienced by investigators ([6-8, 20]) in preparing colloidal solutions of cholesterol were overcome by adding lecithin to the media ([12, 20, 23]). The solubilization of neutral lipophilic molecules in water has been studied in detail ([23, 28]) and the results made possible the preparation of clear colloidal solutions of cholesterol of the order of 0.2 mg per ml ([8]). Cholesterol was usually dissolved in acetone and dispersed in the medium. In the cholesterol-lecithin medium ([12]) used in our experiments, cholesterol was kept in a colloidal solution by the combined effects of yeast extract and lecithin. By increasing the concentration of yeast extract to 4 gm per liter medium, cholesterol remained in colloidal solution after sterilization in an autoclave. However, some precipitation occurred when serum was added to the media. The incorporation of lecithin in the media permitted the obtaining of fine stable colloidal suspensions of cholesterol even when the media were enriched with serum. Fleischer and Brierley ([8]) have shown that "phospholipid micelles provide an elegant vehicle for solubilizing cholesterol."

It is not clear whether lecithin is a further nutrient in our media. Boissevain and Schultz ([4]) found that the growth enhancing property of egg yolk resided in part in the phospholipid fraction. Dubos ([5]) demonstrated that one of the phospholipids in egg yolk protected acid-fast bacteria from toxic factors in the media. A possible similar effect of lecithin in our media remains to be investigated.

The relation of the cultures obtained to the pathologic changes of murine leprosy is not yet clear. Ogawa ([25]) found that the strains he isolated from murine leprous tissues produce the specific disease in mice. We are presently investigating the virulence of the cultured bacilli for the susceptible hosts, rats and mice, as well as the biochemical characteristics of our cultures. Differences will certainly be found between the characteristics of the host-grown *M. lepraemurium* and the strains of mycobacteria cultivated from murine lepromas. There is evidence of such differences when an organism passes from the *in vivo* (Phase I) to the *in vitro* (Phase II) growth phase when its growth conditions are radically changed ([1, 11, 14]). As long as these problems are not clarified, we are unable to claim the successful cultivation of *M. lepraemurium,* nor can we exclude that the strains isolated by Ogawa or by us are identical with the etiologic agent of murine leprosy.

SUMMARY

In vivo grown *M. lepraemurium* suspensions were inoculated into a basal medium containing cholesterol and lecithin. Slow growing strains of mycobacteria were cultured regularly in these media. The presence of free cholesterol or cholesterol in serum or cholesterol in trypsin-digested egg yolk was essential for growth. The primary cultures were difficult to obtain, but the strains were easily subcultured. A heavy inoculum was necessary to obtain primary cultures in the liquid media, no growth occurred on semisolid agar slants. Similarly slow-growing primary cultures were obtained on Ogawa egg yolk media. Growth developed in a considerably shorter time if Ogawa's medium was enriched with 0.4% yeast extract (Difco). The cultures obtained on Ogawa egg yolk media were successfully subcultured in liquid cholesterol-lecithin media. The relation of the cultured strains of mycobacteria to the pathology of murine leprosy is not yet clear. The dynamics of cholesterol metabolism in the macrophages related to murine leprosy is discussed.

RESUMEN

Un medio basal conteniendo lecitina y colesterol se inoculó con suspensiones de *M. leprae-*

murium crecido *in vivo*. En este medio se pudieron cultivar regularmente cepas de micobacterias de crecimiento lento. Para el crecimiento, fue esencial la presencia de colesterol libre, del colesterol sérico o del colesterol contenido en la yema del huevo digerida con tripsina. Los cultivos primarios fueron difíciles de obtener pero las cepas fueron facilmente subcultivables. Para obtener los cultivos primarios en el medio líquido fue necesario un inóculo grande. No se logró crecimiento alguno en agar semisólido. También se obtuvieron cultivos primarios de crecimiento lento similares en el medio de Ogawa con yema de huevo. El crecimiento se logró en un tiempo considerablemente más corto si el medio de Ogawa estuvo suplementado con extracto de levadura (Difco) al 0.4%. Los cultivos obtenidos en medio de Ogawa con yema de huevo se pudieron subcultivar exitosamente en medio líquido con colesterol y lecitina. La relación de las cepas micobacterianas cultivadas con la patología de la lepra murina todavía no esta clara. Se discute la dinámica del metabolismo del colesterol en los macrófagos en relación a la lepra murina.

RÉSUMÉ

Des suspensions de *M. lepraemurium* cultivés *in vivo* ont été inoculées dans un milieu de base contenant du cholestérol et de la lecithine. Des souches de mycobactéries à croissance lente ont été cultivées régulièrement dans ces milieux. La présence de cholestérol libre ou de cholestérol en solution dans du sérum, ou de cholestérol mélangé à du jaune d'oeuf digéré par la trypsine, était essentielle pour la croissance. Il a été difficile d'obtenir des premières cultures, mais les souches étaient ensuite facilement cultivées par passage secondaire. Un inoculum massif était nécessaire pour obtenir les cultures initiales en milieux liquides, car aucune croissance n'a été notée sur des frottis inclinés sur agar semi-solides. Des cultures primaires similaires de bactéries à croissance lente ont été obtenues sur le milieu au jaune d'oeuf d'Ogawa. La croissance s'est manifestée après un délai remarquablement plus court lorsque le milieu d'Ogawa était enrichi avec un extrait de levure à 0,4% (Difco). Les cultures obtenues sur milieu au jaune d'oeuf d'Ogawa ont été sous-cultivées dans des milieux à base de lécithine-cholestérol liquide. La relation entre les souches de mycobactéries ainsi cultivées et la pathologie de la lèpre murine n'est pas encore éclaircie. La dynamique du métabolisme du cholestérol dans les macrophages est discutée dans ses rapports avec la lèpre murine.

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