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Cholesterol, a Factor Which is Required for Growth of Mycobacteria from Leprous Tissues [1, 2]

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Strains of the *M. scrofulaceum* group were originally isolated from human, armadillo *(Dasypus novemcinctus* Linn.) and rat leprous tissues in a basal medium containing glycerol and yeast extract, enriched with serum. The cultures soon became adapted to the culture media on which cultivable mycobacteria were grown ([11]).

The strains did not multiply in the foot pads of mice as *M. leprae* do. Heat killed suspensions of these strains induced Arthus type reactions in tuberculoid as well as in lepromatous leprosy patients. The strains, however, retained some of the genetic markers of *M. leprae*: they were slow growers and gave positive fluorescence with specific *M. leprae* sera. The strains were resistant to all of the antituberculous drugs which have no therapeutic effect against leprosy and were highly sensitive to rifampicin, the most effective drug for leprosy. They were reasonably sensitive to diaminodiphenylsulfone, the slow-acting drug of choice against the disease ([12]). These observations led us to suspect that *M. leprae* might have quite different characteristics when grown in the host as phenotype I from those shown *in vitro* as phenotype II ([10]). This phenomenon is well documented for cultivable mycobacteria ([8]).

For the cultivation of mycobacteria isolated from human and armadillo leprous tissues, the basal medium did not support growth in the absence of serum ([11]). This observation indicated that serum might contain an essential growth factor. Further, the presence of a complex material such as serum in the medium complicates the investigations of growth requirements.

It was, therefore, necessary to search for a well defined factor as an essential growth requirement for the hitherto noncultivable mycobacteria. A study was undertaken to identify the growth factor present in the serum, on the assumption that a well defined growth factor added to the medium might improve the frequency of positive cultures, accelerate growth and lead to the cultivation of strains with a high homology or identity with *M. leprae.*

Findings presented in this paper indicate that cholesterol (CH) is an essential growth factor for isolating mycobacteria from human and armadillo leprous tissues. Results have been previously reported in a note ([9]).

MATERIALS AND METHODS

In vivo grown M. leprae. Armadillos were inoculated with *M. leprae* from human leprous tissues by Dr. O. K. Skinsnes at the Department of Pathology, ALM Leprosy Atelier, University of Hawaii. The animals were sacrificed 18 and 21 months later. Within three days, *M. leprae* bacilli were isolated by differential centrifugation ([11]) from the subcutaneous nodules, liver and spleen. The partially purified suspensions of *M. leprae* were used as inocula into the culture media.

One armadillo was captured in the wild in the state of Louisiana, U.S.A. The animal presented a disseminated mycobacterial infection resembling leprosy. After six months in captivity, the animal was sacrificed by Dr. G. P. Walsh at the Gulf South Research Institute, New Iberia, Louisiana. Leprous tissues were transported on ice within 24 hours to our laboratories. Acid-fast bacilli, probably *M. leprae,* were isolated as above.

In vitro grown strains of mycobacteria. The following strains were used: *M. scrofulaceum* strain Dakar, cultivated from a leprous nodule of a lepromatous leprosy (LL) patient; *M. scrofulaceum* strain A6, cultivated from an armadillo previously infected with *M. leprae* from human LL nodules; *M. scrofulaceum* strain R56, cultivated from a subcutaneous leproma of a rat infected with

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the Hawaiian strain of *M. lepraemurium;* and *M. scrofulaceum* isolated from the sputum of a patient with pulmonary disease.

The cultures were maintained in a yeast extract, glycerol, sheep serum liquid medium, transferring the subcultures twice monthly ([11]).

The culture media. The basal medium (BM), as described by Kato and Ishaque ([11]), contained KH_2PO_4 (8.5 gm), glycerol (30 gm) and yeast extract (Difco, 2 gm) in one liter of distilled water. Nine milliliters of the medium were distributed into each of a series of 50 ml screw cap tubes and sterilized in an autoclave for 30 minutes at 15 lbs pressure. To each tube of BM, 1 ml of filter sterilized sheep serum was added.

Penicillin G sodium was added (200 U/ml) to all media prior to inoculation.

A slight precipitate occurred in the CH containing medium when sheep serum was added. This difficulty was overcome and a precipitate-free medium was obtained by stabilizing the colloidal CH solution with lecithin.

The cholesterol-lecithin medium. Two hundred milligrams cholesterol (USP) were dissolved in 4 ml warm acetone. This solution was injected into one liter BM containing 4 gm yeast extract (Difco). The medium was autoclaved for ten minutes with a loose cotton stopper to evaporate the acetone. Two hundred milligrams of lecithin were carefully homogenized in 20 ml of 0.1 M phosphate buffer, pH 5.8, and this solution was injected with a syringe into the BM containing CH while mixing with a magnetic stirring bar. Nine milliliters of the medium were distributed into each of a series of 50 ml screw cap tubes and sterilized for 25 minutes in an autoclave. One milliliter of filter sterilized sheep serum was added aseptically to each tube.

Decontamination of host grown M. leprae suspensions. A semipurified suspension of host (armadillo) grown *M. leprae* was centrifuged at 8,000 rpm for ten minutes. The sediment was homogenized in approximately 50 times its volume of 4% NaOH and 2.9% sodium citrate solution, mixed in equal volumes. After 20 minutes of incubation at 34°C with occasional shaking, the suspension was centrifuged for ten minutes at 8,000 rpm at 4°C. The sediment was washed twice with sterile 0.1 M phosphate buffer, pH 5.8.

Serum fractions. Lipid free serum (LFS) and serum lipids (SL) were prepared from filter sterilized sheep serum as described by Hartley ([6]). LFS and SL were collected separately.

Preparation of media with cholesterol ([9]) or lipid fractions. One hundred milligrams of cholesterol or lipids (SL) were dissolved in 2 ml of warm acetone or alcohol. From these solutions the amount equivalent to the original volume of serum was injected into the basal medium using a syringe with the point of the needle below the surface of the warm medium. CH and the lipid fractions did not flocculate or precipitate in the basal medium containing 4 gm per liter yeast extract. Each 50 ml screw cap tube contained 9 ml of the medium which remained somewhat opaque but transparent after sterilization in an autoclave for 30 minutes. Filtered sterilized sheep serum was added to the media as indicated below.

Semisolid media were prepared by adding 1.5% agar (Difco) to the BM containing CH or the SL. Following sterilization in an autoclave for 30 minutes, agar slants were prepared with or without aseptically added serum or LFS.

Growth and multiplication. Cultures were incubated at 34°C. Criteria for growth and multiplication have been previously described ([11]). For all practical purposes a slight increase in the number of acid-fast bacilli in the media was disregarded. The progress of growth was designated as:

—	When no multiplication of the inoculum occurred as estimated microscopically in stained preparations.
±,+	When apparent multiplication occurred.
++,+++	Easily estimated multiplication.
++++	Good growth.
++++ ++++	Heavy growth, the number of bacilli in stained preparations being so heavy as to preclude the counting of acid-fast units.

Growth was estimated on the surface of the agar slants by the appearance and consistent growth of colonies, confirmed by microscopic examination of the bacilli which formed the colonies.

Heavy inocula were used to obtain the isolation-cultures and for subculturing the primary cultures. Criteria for obtaining positive subcultures were the same as for the isolation-cultures.

RESULTS

Growth with lipid fractions of sheep serum. A limited multiplication of acid-fast bacilli developed in the BM enriched with 10% sheep serum 30 days after inoculation with mycobacteria isolated from an armadillo infected with *M. leprae* and from leprous tissues of another armadillo captured in the wild.

In the BM, lipid free serum (LFS) resuspended in saline to the original volume of the serum or serum lipids (0.2 mg/ml) failed to promote growth. When both LFS and SL were incorporated in the BM, bacilli grew well in the media inoculated with the bacilli from either of the two leprous animals. Bovine serum albumin (BSA) 10% v/v in the BM with the serum lipids gave somewhat better growth as compared to sheep serum or lipid free serum. Both strains gave an excellent growth in the BM enriched with both the serum lipids and the complete sheep serum. One milliliter of the cultures obtained from both strains was transferred after 30 days of incubation into the homologous medium. Strains grew well in the subsequent several subcultures within ten days of incubation. Results are shown in Table 1.

Growth with cholesterol with or without lecithin added. When 0.2 mg/ml of CH was incorporated into the BM with no serum added, multiplication of acid-fast bacilli did not occur during 30 days of incubation. In BM containing 0.2 mg/ml CH and enriched with either LFS or BSA, a considerable multiplication was observed in 30 days. Bacilli from both armadillos increased in number within ten days in media containing CH added to the BM enriched with 10% sheep serum. The cultures advanced to a heavy growth of acid-fast bacilli in 30 days. The strains were easily subcultured in the homologous medium and are maintained in the same medium by serial transfers at two week intervals. Results are shown in Table 1.

Similar results were obtained in the CH-lecithin media enriched with sheep serum. Lecithin without CH did not promote growth. The use of lecithin in the medium was advantageous because of the absence of CH flocculation.

Growth characteristics and morphology of the bacilli in media containing cholesterol. Growth of the initial inoculum in the yeast extract-glycerol-CH-sheep serum medium developed as a somewhat yellowish sediment with an opalescent supernatant. When the cultures were shaken, many microcolony-like formations were visible in the suspension. Bacilli were weakly acid-fast during the beginning of growth, but became strongly acid-fast with progressive growth.

The inoculum as separated from the leprous tissues consisted of host grown acid-fast bacilli arranged in bundles or microcolonies typical for suspensions of *M. leprae* isolated

TABLE 1. *Effects of serum fractions added to the yeast-extract glycerol basal medium (BM) on the growth of mycobacteria isolated from two armadillos.*

Substances added to the BM	Inoculum from armadillo infected with *M. leprae*				Inoculum from armadillo, infected naturally with *M. leprae* (?)			
	Growth in days			Growth in 10 days in subcultures	Growth in days			Growth in 10 days in subcultures
	10	20	30		10	20	30	
Se 10%	+	+	++		+	±	+	
LFS 10%	—	—	—		—	—	—	
SL 0.2 mg/ml	—	—	—		—	—	—	
SL + LFS	—	—	+		—	+	++	
SL + BSA 10%	—	—	+++		—	+	++++	
SL + Se	—	—	+++	+++	±	++	++++	++++
CH 0.2 mg/ml	—	—	—		—	—	—	
CH + LFS 10%	—	±	++		+	++	+++	
CH + BSA 10%	±	+	+++		+	++	+++	
CH + Se 10%	+	++	++++ ++++	++++ ++++	+	++++	++++ ++++	++++ ++++
Lowenstein-Jensen medium	—	—	—	—	—	—	—	—

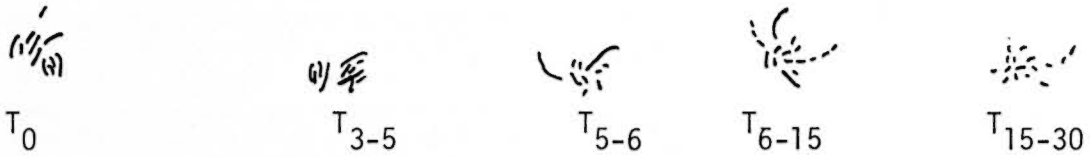

FIG. 1. Schematic representation of growth morphology of mycobacteria from leprous tissues in cholesterol media during 30 days of cultivation (T_0 to T_{30}).

from LL patients or leprous armadillos. These bacilli retained the same arrangements in groups with a few singular bacilli in the culture media. Within three to five days some bacilli, in most of the bundles or microcolonies, became elongated reaching up to 20 times the length seen at the time of inoculation. More and more bacilli became similarly elongated reaching out, but remaining attached to the bundles or microcolonies. Then quite suddenly (i.e., overnight) the long units became segmented and more and more strongly acid-fast bacilli or coccoid bacilli were often seen still attached or close to the bundles or microcolonies, often disengaged from the groups. The heavy inoculum lost more and more its bundle and microcolony appearance with the process of elongation, fragmentation and multiplication advancing. As growth became heavier and heavier, the bundles and microcolonies tended to disappear leaving the impression that the heavy growth was the direct result of elongation and multiplication of individual cells from the bundles and globi (Fig. 1).

In the CH containing liquid media, growth varied from fine granularity to more definite small floating colonies, or homogenous suspensions, depending on the size of inoculum or the frequency of shaking of the cultures.

On the surface of the CH, or the CH-lecithin containing semisolid media, enriched with 10% sheep serum, visible growth occurred within three to seven days when inoculated with any of the *in vitro* grown strains. Colonies were first transparent, turning opaque and only aging cultures formed a light yellowish pigment. The same type of colonies developed on the semisolid media when inoculated with heavy suspensions of leprosy bacilli isolated from the armadillos, however, it took 15 to 45 days for growth to become visible. Bacilli grown on the semisolid surfaces were nonacid-fast at first but became strongly acid-fast as growth of the colonies progressed.

Effect of serum with cholesterol on growth. Different batches of sheep serum and sera of different species were not equal in promoting *in vitro* growth of the tested strains of mycobacteria. However, when CH (0.2 mg/ml) was added to the BM, the growth promoting effect of sera was independent of the quality of different batches of serum or the species of origin. Results are shown in Tables 5 and 6. Again the use of the CH-lecithin media was advantageous due to the absence of precipitate with sera added.

Source of carbon. CH (0.2 mg/ml) was added to the BM and enriched with 10% sheep serum. All of the four *in vitro* grown strains of mycobacteria as well as mycobacteria isolated from the leprous tissues of the *M. leprae* infected armadillo grew abundantly within five days in the media. When the same media were prepared with glycerol deficiency, only *M. scrofulaceum* grew vigorously in the absence of glycerol, while there was no growth in the culture media inoculated with mycobacteria from armadillo leprous tissues, or the *in vitro* grown strains cultivated from human (M. Dakar), armadillo (M.A6) or rat (M.R56) leprous tissues. CH was not a source of carbon for any of the tested mycobacteria from tissues or from cultures, except for *M. scrofulaceum*. Results are shown in Table 7.

The effects of varying amounts of CH in the BM on the growth of mycobacteria isolated from the two armadillos are shown in Table 2. This was investigated in media enriched with sheep serum, since results shown in Table 1 indicated that CH did not promote growth in the absence of serum proteins. A minimum concentration of 0.1 mg/ml of CH was necessary to obtain an enhanced multiplication of mycobacteria. With increased amounts of CH the growth was not further augmented. It seems that the highest concentration (1.0 mg/ml) used in this experiment had less growth promoting effect than 0.1 to 0.5 mg/ml CH. Similar results were obtained when the *in vitro* grown strains M.

Dakar, M.A6, M.R56 and *M. scrofulaceum* were cultivated in the presence of increasing amounts of CH in the BM, as shown in Table 3. Lecithin added to the CH containing media did not influence the rate of growth. Since lecithin prevented the aggregation of CH with the serum proteins, the visual estimation of bacillary multiplication was greatly facilitated.

Mycobacteria separated from the leprous tissues of an *M. leprae* infected armadillo and bacilli from the four *in vitro* grown strains of mycobacteria were inoculated into the CH containing BM with varying amounts of sheep serum respectively. Growth was estimated at different time intervals during incubation at 34°C. Results are summarized in Table 4. No growth was observed in the absence of serum. Without CH in the media, increasing amounts of serum yielded an increased growth. When a constant amount of 0.2 mg/ml of CH was incorporated in the media, even a small amount of 2% serum permitted the same rate of growth as that of the highest serum concentration tested (10%). Thus, in the absence of CH, the growth rate was dependent on the serum concentration, while in the presence of CH, serum was needed for growth but the growth rate was independent of the serum concentration in the range of 2-10%.

DISCUSSION

Cholesterol is the principal sterol of higher animals and an ubiquitous constituent of living cells. CH is one of the major lipid components associated with serum lipoproteins. The ubiquitous presence of CH in animal cells has attracted a great deal of interest. Despite efforts to uncover its physiologic

TABLE 2. *Effects of varying amounts of cholesterol in the basal medium containing yeast extract, glycerol and sheep serum on the growth of mycobacteria isolated from two armadillos.*

Concentration of cholesterol mg/ml	Inoculated from an armadillo, infected with *M. leprae.* Growth in days			Inoculated from an armadillo, infected naturally. Growth in days		
	10	20	30	10	20	30
1.0	+	++	+++	++	+++	++++
0.5	++	++++	++++ ++++	++	++++	++++ ++++
0.25	+	++++	++++ ++++	++	++++	++++ ++++
0.1	+	++	++++ ++++	++	+++	++++ ++++
0.01	—	+	++	—	—	++
0.00	±	+	++	+	+	+++

TABLE 3. *Effects of varying amounts of cholesterol in the basal medium containing yeast extract, glycerol and sheep serum on the growth of* in vitro *grown strains of mycobacteria isolated from human, armadillo and rat leprous tissues.*

Concentration of cholesterol mg/ml	Growth in 10 days			
	M. Dakar	M.A6	M.R56	*M. scrofulaceum*
1.0	++++	++++	+++	++++
0.5	++++ ++++	++++ ++++	++++ ++++	++++ ++++
0.25	++++ ++++	++++ ++++	++++ ++++	++++ ++++
0.1	++++ ++++	++++ ++++	++++	++++ ++++
0.01	+++	+++	++	+++
0.00	+++	++++	++	++++

significance and its role in pathologic processes, our knowledge regarding the function of CH in cell metabolism is still limited. This molecule participates as such in cell structures or in some physicochemical mechanisms. It was quite early suspected that some animal cells might synthesize, some others might decompose CH, however, these processes were difficult to investigate because of the relative insolubility of this compound in water or tissue fluids.

Sohngen in 1931 ([19]) was the first to report that various species of mycobacteria were able to grow in a medium with CH as the sole source of carbon. Similarly, Haag ([19]) noted that the genus *Mycobacterium* multiplied in a medium in which CH was the sole organic substance. He described two strains of mycobacteria, *M. testudo* and *M. lacticola*, which grew abundantly in CH containing medium. Tak ([19]) reinvestigated the problem of bacterial decomposition of CH

TABLE 4. *Effects of varying amounts of sheep serum with and without cholesterol (CH) (0.2 mg/ml) in yeast extract (0.4%), glycerol (3%) containing media on the growth of mycobacteria from an* M. leprae *infected armadillo, of strains cultivated from leprous tissues and on* M. scrofulaceum.

Inoculum / % v/v sheep serum	*M. leprae* infected armadillo				M. Dakar		M.A6		M.R56		*M. scrofulaceum*	
	Growth in days											
	20 no CH	30 no CH	20 CH	30 CH	10 no CH	10 CH	10 no CH	10 CH	10 no CH	10 CH	10 no CH	10 CH
0	—	—	—	—	+	+	+	+	—	—	++	++
2	—	—	+	+	++	++++ ++++	++	++++ ++++	±	++++	+++	++++ ++++
4	—	+	++++	++++ ++++	+++	++++ ++++	+++	++++ ++++	+	++++	+++	++++ ++++
6	—	+	++++	++++ ++++	++++	++++ ++++	++++	++++ ++++	+++	++++	++++	++++ ++++
8	±	++	++++	++++ ++++	++++	++++ ++++	++++	++++ ++++	++++	++++	++++ ++++	
10	+	++	++++	++++	++++	++++ ++++	++++	++++ ++++	++++	++++	++++	

TABLE 5. *Effects of cholesterol (0.2 mg/ml) in yeast extract-glycerol basal medium supplemented respectively with different batches of serum (10% v/v) on the growth of mycobacteria isolated from an armadillo infected with* M. leprae.

10% v/v Serum added to basal medium	Without added cholesterol			With cholesterol added		
	Growth in days					
	10	20	30	10	20	30
None	—	—	—	—	—	—
Human	—	—	±	+	+++	++++
Horse	—	—	+	+	+++	++++
Bovine	—	—	++	—	++	+++
Sheep I	—	++	++++	++	++++	++++ ++++
Sheep II	—	—	+	++	++++ ++++	++++++ ++++++
Sheep III	—	—	—	+	++	++++
Sheep IV	—	+++	++++	+	+++	++++ ++++
Goat	+	++	++++	++	++++	++++
Rabbit	—	—	—	+	+++	++++

and searched systematically for species adapted to the task of CH decomposition. Tak prepared fine suspensions of CH with yeast autolysate. He isolated several cultures from soil which consumed CH in the media. This author identified the obtained "cholesterol decomposers" as *M. lacticola, M. berolinense, M. cholesterolicum* n. sp. Tak pointed to the fact "isolation of active cholesterol decomposers has led in all cases to species of the genus *Mycobacterium*. For, it is exactly for representatives of this genus that both Sohngen and Haag have claimed the property of cholesterol decomposition." Tak also found that in cultures of *M. phlei, M. salmonicolor* and *M. rubrum* "cholesterol disappeared with relatively great speed" from media in which CH was the only source of carbon. Bleecken and Schubert ([3]), using tritiated CH in autoradiographic technics, reported the "surprisingly fast utilization of cholesterol" by mycobacteria. Many L phase bacteria are able to synthesize CH ([4]). Pleuropneumonia group organisms (mycoplasma) require CH for growth ([4,5]) and in this respect they resemble the mycobacteria

TABLE 6. *Effects of different pooled batches of sheep sera with and without cholesterol (0.2 mg/ml) added to the basal medium on the growth of strains of mycobacteria isolated from leprous tissues and* M. scrofulaceum.

Basal medium	Batches sheep serum 5% (serum-cholesterol mg %)	Strains of mycobacteria growth in 14 days			
		M. Dakar	M.A6	M.R56	*M. scrofulaceum*
Without cholesterol	Apr. 1976 (106)	+	++	±	++
	Sept. 1976 (194)	++	++++	++	++++
	Dec. 1976 (260)	++++	++++ ++++	+++	++++ ++++
	May 1977 (99)	+	++	+	+++
	Sept. 1977 (88)	+	++	±	+++
With cholesterol 0.2 mg/ml	Apr. 1976 (106)	++++	+++ +++	+++	++++ ++++
	Sept. 1976 (194)	++++	++++ ++++	+++	++++ ++++
	Dec. 1976 (260)	++++	++++ ++++	++++	++++ ++++
	May 1977 (99)	++++	++++ ++++	+++	++++ ++++
	Sept. 1977 (88)	++++	++++ ++++	+++	++++ ++++

TABLE 7. *Effects of cholesterol (0.2 mg/ml) in yeast extract (0.4%) sheep serum (10% v/v) containing media with and without glycerol (3%) added on the growth of mycobacteria from an* M. leprae *infected armadillo, of strains cultivated from leprous tissues and on* M. scrofulaceum.

Inoculum / Medium	*M. leprae* infected armadillo		M. Dakar		M.A6		M.R56		*M. scrofulaceum*	
	Growth in days									
	20	30	5	10	5	10	5	10	5	10
Yeast extract sheep serum cholesterol	—	—	+	+	+	++	+	+	++++ ++++	++++ ++++
Same with glycerol 3% added	++++	++++	++++	+++++	++++	++++	+++	++++	++++ ++++	++++ ++++

isolated from leprous tissues. Such a correlation was already reflected in the experiments of Barksdale *et al* [1].

Failure to grow *M. leprae* and *M. lepraemurium in vitro* is due to the peculiar properties of the organisms, especially their exacting cultural requirements. *M. leprae*, however, multiplies abundantly in the macrophages which fulfill growth requirements. CH metabolism in the macrophage was extensively studied by Werb [22]. In carefully controlled experiments, Werb established the "Dynamics of macrophage membrane cholesterol." CH plays an important role in the metabolism of macrophages [22,25]. Werb and Cohn [23] have shown that macrophages are rich in free CH and that more than 35% of the total CH was membrane associated. Macrophages can synthesize CH from acetate-1C [23]. Macrophages are provided with a peculiar mechanism permitting the rapid exchange of membrane CH with serum lipoprotein. The exchange is independent of bulk transport of lipoproteins by phagocytosis and is not linked to energy metabolism. Werb and Cohn [23] provided evidence for the lysosomal membrane and plasma membrane localization of CH compartments. CH content and phospholipid content of the secondary lysosome and intracellular membranes increased with extensive pinocytosis of nondegradable particles [24]. These experimental models of Werb and Cohn are comparable to the pathology of lepromatous leprosy where the *M. leprae* is a "nondegradable particle" in the macrophage of the susceptible host.

"Under physiological and pathological conditions, macrophages are exposed to many forms of free and esterified CH. Erythrocytes, tissue cells and chylomicrons are taken up by phagocytosis and subsequently are localized within cytoplasmic phagolysosomes" [15]. The processing and fate of CH within the digestive vacuole was studied by Werb and Cohn [24]. Liposomal CH esterase was the enzyme which hydrolyzed CH with an optimal activity at pH 4. Free CH was formed from CH linoleate and palmitate with accumulation of free CH intralysosomally.

Kondo and Kanai [13,14,16] demonstrated that *in vivo* grown mycobacteria were closely associated with host CH. Investigations of these authors suggest that the infecting mycobacteria might esterify tissue cell CH or CH esters of host origin are adsorbed onto the surface of the infecting mycobacteria [13]. CH esters accumulated in macrophages incubated with mycobacteria *in vitro* [14]. Mycobacteria had ample opportunity to take up CH since Kondo and Kanai found that in the mouse peritoneal cavity, casein induced and noninduced macrophages contained a high level of CH [13].

Both Werb and Cohn [22,25] and Kondo and Kanai [8,13,16] provided important data on CH content, CH transport and CH metabolism in macrophages. Since the macrophage is the *in vivo* culture medium for *M. leprae* and since susceptibility to leprosy lies either in the inability of the macrophage to destroy *M. leprae* and/or to provide factors for the multiplication of *M. leprae*, the recent data emerging from the experiments of Werb and Cohn as well as those of Kondo and Kanai should be carefully observed by students of leprology.

"Thus, there is probably available to the macrophage-engulfed mycobacterium a large amount of cholesterol" [1]. Bacteria, in general, when in the presence of CH take it up [21] and mycobacteria are able to assimilate [18,20] and to esterify CH with fatty acids [17]. Thus, CH readily available in the macrophage might serve as a growth factor for the multiplication of *M. leprae* in the susceptible host. For the *in vitro* grown mycobacteria isolated from leprous tissues, it was CH which supported growth in media enriched with serum as evidenced by the results that lipid free serum did not permit growth, and CH alone or CH plus serum albumin, or CH plus lipid free serum satisfactorily replaced serum for growing mycobacteria from leprous tissues. CH can definitely be considered as a growth factor since it has not been possible to obtain growth of these mycobacteria except in media containing CH in serum or chemically defined CH [10].

As early as 1918, workers used CH as an ingredient in culture media [18-20]. Tak [19] reported that Haag in 1918 and Sohngen in 1931 cultivated mycobacteria from soil with CH as the sole source of carbon in the media. The charcoal agar medium of Hirsch [7] contained free CH in a colloidal dispersion. Barksdale *et al* [2] isolated L form microorganisms from leprous tissues in a medium containing CH. Kondo and Kanai [15] at-

tempted to cultivate mycobacteria in a simple synthetic liquid medium containing lecithin-CH liposome.

Bacteriologic media presently used for the isolation of mycobacteria contain, without exception, organic material such as serum, serum albumin, bovine serum albumin fraction V, whole egg or egg yolk. Each of these ingredients contains considerable amounts of CH, reaching 300 mg% in animal serum and 1.6 gm per 100 gm in egg yolk. Without these CH containing ingredients, none of the pathogenic species of mycobacteria can be isolated from sputum, gastric lavage, urine, feces or other specimens. One is thus tempted to consider CH as the common growth factor in these media. Only when adapted to *in vitro* life as phenotype II will the pathogenic and the saprophytic mycobacteria grow in the absence of CH containing ingredients. The sterol is not a carbon source since no growth occurred in media with CH as the only source of carbon or in the absence of glycerol.

The obtained cultures of mycobacteria are related to leprosy in as much as they were cultivated in CH containing media from suspensions of *M. leprae* isolated from leprous tissues. The strains of mycobacteria grown in the presence of CH tend to form globi-like microcolonies, so characteristic of the appearance of *M. leprae* in the host cells. Whether the strains are identical with *M. leprae*, the established etiologic agent of leprosy, can neither be claimed nor can it be excluded. When an organism passes from the *in vivo* (Phe I) to the *in vitro* (Phe II) growth phase, its growth conditions and growth requirements are radically changed ([1,8,9]). Competition for host constituents and with host defense is, of course, not a multiplication inhibiting factor in culture media. In the primary isolation culture, carry-over of host factors will help to promote growth. There are indications that in the host CH might be a growth factor ([13-16]). If so, it is the same well-defined chemical entity—CH—which is probably the essential growth requirement in the host as well as in the test tube. *In vitro* grown organisms become more exact in substrate requirement which, if not fulfilled, will make subculturing difficult or result in failure ([1,8]). Success of subcultures will depend on adaptation to the new substrates, or better if organisms are offered *in vitro* the same growth factor which promoted growth *in vivo*. CH is readily available in the macrophage for the leprosy bacilli and mycobacteria have the ability to pick up CH ([14,17-20]), and no growth occurred *in vitro* in our experiments without CH. These results suggest that our strains of mycobacteria utilized the same molecule—CH—for *in vivo* as well as for *in vitro* multiplication.

Fortunately, an oxidizable substrate for *M. leprae*, yeast extract ([11]), permitted the incorporation of stable colloidal suspensions of the water insoluble CH into a culture medium ([10]). Lecithin, in equimolar concentration with CH in the medium, formed a colloidal solution with the added ingredients; yeast extract and glycerol. The medium is easy to prepare and can be sterilized in an autoclave. No precipitate occurs when serum is added to the CH-containing medium in the presence of lecithin; an important practical feature of the medium.

The obtained strains of mycobacteria await further characterization. The use of the CH-lecithin containing media in cultivation trials for *M. leprae* is now under further investigation.

SUMMARY

In a yeast extract, glycerol and sheep serum containing medium, slow but abundant growth of mycobacteria occurred when media were inoculated with *M. leprae* isolated from leprous tissues of armadillos (*Dasypus novemcinctus* Linn.). The lipid fraction of the serum was the essential factor for growth. Cholesterol not only replaced, but surpassed the growth promoting effect of the lipid fraction. However, growth of mycobacteria was observed only when media were enriched with serum. The relationship of the obtained strains of mycobacteria to leprosy is not yet clear.

The following cholesterol medium, stabilized with lecithin, is proposed for primary cultivation of mycobacteria from leprous tissues: KH_2PO_4—8.2 gm, Na_2HPO_4—0.5 gm, yeast extract (Difco)—4 gm, and glycerol 30 gm, dissolved to make one liter basal medium in distilled water. Cholesterol (200 mg) dissolved in 4 ml warm acetone is injected with a syringe into the basal medium. The solution is autoclaved for ten minutes to evaporate the acetone. Lecithin, 200 mg dissolved in 20 ml of the basal medium is mixed to the

medium cooled to room temperature. Nine milliliter aliquots are distributed into each of a series of 50 ml screw cap tubes and autoclaved for 25 minutes. One milliliter of filter sterilized sheep serum is added to each of the tubes containing 9 ml of the cholesterol-lecithin medium.

Semisolid media are prepared the same way but 1.5% agar w/v is added to the cholesterol-lecithin medium before autoclaving. When cooled to 56°C, 10% w/v sheep serum is mixed to the liquid. The medium is distributed into screw cap tubes and agar slants are poured and allowed to solidify in the inclined tubes at room temperature.

Macrophages contain considerable amounts of cholesterol. Cholesterol is proposed as a possible growth factor for host grown *M. leprae* in the macrophages of the susceptible host and the same sterol as a growth factor for primary cultivation of mycobacteria from leprous tissues.

RESUMEN

Utilizando un medio a base de extracto de levadura, glicerol y suero de borrego, se logró el crecimiento, lento pero abundante, del *M. leprae* aislado de los tejidos leprosos de armadillos (*Dasypus novemcinctus,* Linn.). El factor esencial para el crecimiento fue la fracción lípida del suero. El colesterol, no sólo reemplazó sino que sobrepasó el efecto promotor del crecimiento mostrado por la fracción lípida. Sin embargo, el crecimiento de las micobacterias sólo se observó cuando los medios se enrriquecieron con suero. Todavía no se ha establecido la relación de las cepas micobacterianas aisladas, con la lepra.

Se propone el siguiente medio con colesterol, estabilizado con lecitina, para el cultivo primario de micobacterias, a partir de tejidos leprosos: KH_2PO_4-8.2 gm, Na_2HPO_4-0.5 gm, extracto de levadura Difco-4.0 gm y glicerol-30 gm, disueltos en agua destilada para hacer 1.0 litro de medio basal. El colesterol (200 mg), disuelto en 4 ml de acetona tibia, se inyecta con jeringa en el medio basal. La solución se somete al autoclave durante 10 min para evaporar la acetona. La lecitina (200 mg disueltos en 20 ml de medio basal) se adiciona al medio enfriado a temperatura ambiente. El medio completo se reparte en alícuotas de 9.0 ml, en tubos de 50 ml con tapón de rosca y se esteriliza al autoclave durante 25 min. Cuando el medio se ha enfriado, se adiciona 1.0 ml de suero de borrego esterilizado por filtración a cada tubo conteniendo 9.0 ml del medio con colesterol y lecitina.

El medio semisólido se prepara de la misma manera pero se adiciona de agar al 1.5% (peso/volúmen) antes de ser esterilizado en el autoclave. Cuando el medio se ha enfriado a 56°C, se adiciona 10% de suero estéril de borrego. El medio se reparte en tubos con tapón de rosca y se deja solidificar en posición inclinada a temperatura ambiente.

Los macrófagos contienen cantidades apreciables de colesterol. El colesterol se propone como un posible factor de crecimiento para el *M. leprae* presente en los macrófagos de los huéspedes susceptibles. El mismo esterol se propone como factor de crecimiento para el cultivo primario de las micobacterias aisladas de tejidos leprosos.

RÉSUMÉ

L'inoculation dans un milieu contenant un extrait de levure, du glycerol, et du sérum de mouton, de *M. leprae* isolé de tissus lépreux d'armadillos (*Dasypus novemcinctus* Linn) permet d'obtenir une croissance lente mais abondante de mycobactéries. La fraction lipidique du sérum constituait jusqu'à présent le facteur essentiel de croissance. Le cholestérol non seulement remplace, mais également surpasse la fraction lipidique en ce qui concerne l'effet promoteur. Néanmoins, cette croissance mycobactérienne n'a été observée que lorsque le milieu est enrichi en sérum. La relation des souches de mycobactérie ainsi obtenues à *M. leprae* n'est pas encore claire.

On propose le milieu suivant à base de cholestérol, stabilisé avec de la lécithine, pour la mise en culture initiale de mycobactéries provenant de tissus lépreux: KH_2PO_4—8,2 gm, Na_2HPO_4—0,5 gm, extrait de levure (Difco)—4 gm, et glycerol 30 gm, dissout de faon a fournir un litre de milieu de base dans l'eau distillée. On injecte alors dans le milieu de base, au moyen d'une seringue, 200 mg de cholestérol dissout dans 4 ml d'acétone chauffée. La solution est ensuite autoclavée pendant 10 minutes afin d'évaporer l'acétone. De la lécithine, à raison de 200 mg dissoute dans 20 ml de milieu de base est alors mélangée au milieu refroidi à la température ambiante. On prépare alors des aliquots de 9 ml que l'on distribue dans des tubes à bouchon à visser d'une capacité de 50 ml, qui sont alors autoclavés pour 25 minutes. On ajoute 1 ml de sérum de mouton filtré et stérilisé, à chacun des tubes contenant 9 ml du milieu cholestérol-lécithine.

Des milieux semi-solides sont préparés de la même façon, mais 1,5% d'agar poids/volume est ajouté au milieu de cholestérol-lécithine avant de passer à l'autoclave. Lorsque le milieu est refroidi à 56°C, 10% poids/volume de sérum de mouton est mélangé au liquide. Le milieu est alors distribué dans les tubes à bouchon à visser, complété par de l'agar, le tout solidifiant alors dans les tubes inclinés à la température ambiante.

Les macrophages contiênnent des quantités considérables de cholestérol. On suggère que le cholestérol est peut-être un facteur de croissance pour *M. leprae* lorsque celui-ci se multiplie dans les macrophages de l'hôte susceptible. On propose de considérer ce même stérol comme un facteur de croissance pour la mise en culture initiale de mycobactéries provenant de tissus lépreux.

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