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Investigations into the cultivation of *Mycobacterium leprae*. A multifactorial approach

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Introduction

Evidence is accumulating that *Mycobacterium leprae* is probably a microbe-dependent microbe.[10–13] The first report that multiplication of *M. leprae* was promoted by a growth factor of mycobacterial origin was proposed by Twort[19] in 1910, two years before the discovery of the cultivation of 'Johne's bacilli' in media enriched with heat-killed mycobacteria.[20] The growth factor was identified 37 years later as mycobactin.[4] It became evident that mycobacteria necessitate two distinct iron transport compounds: exochelins for extracellular iron acquisition and mycobactins for cell-wall iron transport and intracellular iron storage.[14,15]

In attempting *in vitro* cultivation of *M. leprae*, the results of Twort[19] remained unnoticed until Hanks[6] adopted 'the mycobactin-requiring *M. paratuberculosis* as a model toward the cultivation of *M. leprae*'. When Ishaque and Kato[7] reported the presence of a cytochrome system in *M. leprae*, iron was recognized as an essential element for *M. leprae*. Consequently, as for all mycobacteria, the iron transport compounds are integral mediators for the functioning of the iron-dependent cytochromes, electron transport and energy metabolism of *M. leprae*.

Hall, Wheeler & Ratledge[5] have shown that exochelins from *M. neoaurum* mediate extracellular iron uptake into *M. leprae*. Due to methodological difficulties, it was not evident whether *M. leprae* could produce its own exochelins. *M. leprae* is, however, deficient in mycobactin, as reported by Kato.[11]

The above data, coupled with the ever-increasing number of reports on the cultivation of leprosy-derived mycobacteria from *M. leprae*-infected human and armadillo tissues, prompted Kato to propose that *M. leprae* was probably a microbe-dependent microorganism[10,11] This concept (Table 1) implies that the cultivable leprosy-derived mycobacteria are an integral part of the pathology, by providing the iron transporting compounds for *M. leprae*.[10–13]

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Table 1. Data leading to the concept that *M. leprae* might be a microbe-dependent microbe

Year	Authors	Pertinent data
1910	Twort[19]	Cultivation of *M. leprae* on media enriched with killed 'Tubercle bacilli'
1912	Twort and Ingram[20]	Cultivation of Johne's bacilli. Concept of a growth factor for mycobacteria
1949	Francis *et al.*[4]	Growth factor identified as mycobactin
1966	Hanks[6]	Microbe dependent mycobacteria: models for growing *M. leprae*
1975	Macham & Ratledge[14]	Iron acquisition of mycobacteria by exochelins
1975	Macham *et al.*[15]	Necessity of two distinct iron transport compounds for mycobacteria: exochelins and mycobactin
1977	Ishaque *et al.*[7]	Cytochrome system present in *M. leprae*: iron is essential
1983	Hall *et al.*[5]	Exochelin mediated iron uptake into *M. leprae*
1873–1986	See[9–11]	Frequent cultivation of mycobacteria from *M. leprae* infected tissues
1984	Kato[10]	Leprosy derived mycobacteria, probably etiological cofactors in leprosy
1985	Kato[1–13]	Absence of mycobactin in *M. leprae*: probably a microbe dependent microbe

Exochelins did not act as a growth factor for *M. leprae* in the experiments of Hall & Wheeler.[5] In full agreement with Hall *et al*,[5] the cultivation of *M. leprae* must be considered 'a multifactorial problem'. In searching for such multifactorial, cooperating factors, contributing to the *in vivo* and *in vitro* growth of *M. leprae*, some data are already available and summarized in Table 2.

Maximal endogenous respiration by host-grown *M. leprae* was registered at pH 5·8.[7] This H-ion concentration, maintained by a PO_4 buffer, is proposed for a prospective culture medium. Ferric ions will be necessary for the functioning of the cytochromes.[7] Endogenous respiration of *M. leprae* was stimulated by sulphydril compounds.[7] For this purpose Na thioglycolate is an ideal candidate in the medium.[1] Yeast extract also stimulated respiration and ATP formation. A chemically well defined, potent growth promoting ingredient in yeast extract (thioctic acid)[16,19] is a promising factor for growing *M. leprae*. Since exochelins mediate iron uptake for *M. leprae*[5] and *M. leprae* does not produce mycobactin,[11] it is logical to incorporate both exochelins and mycobactins in a prospective culture medium for *M. leprae*. It is obvious that exochelins and mycobactins produced by leprosy-derived mycobacteria (LDM) should be used.

Ishaque & Kato[7] found the cytochromes in a reduced state in *M. leprae*, suggesting reduced O_2 requirements for *M. leprae* and probably toxic effects of

Table 2. Data pertinent to the formulation of prospective multifactorial culture media for *M. leprae*

Data on *M. leprae*	Proposed constituents in media
Ishaque & Kato[8]—Optimal endogenous respiration pH 5·8. Cytochrome system present. Iron needed. Respiration stimulated by SH compound. ATP formation stimulated by yeast extract	M/15 PO_4 buffer pH 5·8, ferric ion, Na thioglycolate, and lipoic (thioctic) acid
Hall *et al.*[5]—Iron uptake mediated by exochelins	Exochelins (for iron uptake) from leprosy derived mycobacteria
Kato[11]—Absence of mycobactin in *M. leprae*	Mycobactins (for intracellular iron transport/storage) from leprosy-derived mycobacteria
Ishaque *et al.*[7]—Cytochromes present in reduced state	Reduced O_2 tension in media
Campbell[2]—Low O_2 concentration in tissues	Microaerophilic conditions
Macham *et al.*[15]	Exochelins and mycobactin in filtered spent Tween 80 media

high O_2 tension. Oxygen concentration is low in tissues.[2,18] Therefore, microaerophilic conditions in media might contribute to growth of *M. leprae.*

The above multifactorial conditions will be considered in proposing media for cultivation trials of *M. leprae.*

Materials and methods

MICROORGANISMS

Mycobacterium leprae cell suspensions were obtained aseptically from spleen or liver of four *M. leprae*-infected armadillos. Partially purified cell suspensions were obtained by differential centrifugation, and were treated with 2% NaOH for 20 min. Following neutralization with HCl, cells were washed with M/15 phosphate buffer pH 6·5 and re-suspended in the liquid medium to obtain approximately 10^7–10^8 acid-fast rods/ml. Bacilli were sent *via* air mail in this medium. Cell suspensions arrived in Montreal in 8–10 days.

M. intracellulare (LDM) (serotype 19) was isolated from an *M. leprae*-infected armadillo.

M. phlei and *M. scrofulaceum* (H1-75) (both LDM) were isolated from lepromata of lepromatous leprosy patients.

M. avium-intracellulare (Lufu), an environmental isolate by F. Portaels.

M. paratuberculosis ATCC 19.698

EXOCHELIN (E) AND EXOCHELIN–MYCOBACTIN (E–M) RICH FILTRATES

Two LDM, *M. intracellulare* and *M. phlei*, were grown respectively in 500-ml flasks containing each 200-ml iron-deficient glycerol-asparagin (Sauton) media. *M. intracellulare* was cultivated for 20 days, *M. phlei* for 10 days at 34°C. Cultures were autoclaved for 45 min and filtered while hot on filter paper. The filtrates were collected and served for the exochelin media.

The filtrates were designated as: IE, E filtrate of *M. intracellulare*; and PE, E filtrate of *M. phlei*.

The two LDM were also grown in the iron-deficient Sauton media containing 1% Tween 80, resulting in the presence of mycobactins—in addition to exochelins in the filtrate according to Macham *et al.*[15] The filtrates were designated as: IEM, E/M filtrate of *M. intracellulare*; and PEM, E/M filtrate of *M. phlei*.

Exochelins and mycobactins were not extracted and purified from the filtrates. When adjusted to pH 6·5 and autoclaved, the filtrates promoted growth of the mycobactin-dependent *M. paratuberculosis* ATCC 19.698.

THE MEDIA

In order to assure the hypothetical multifactorial growth requirements for *M. leprae*, data of Table 2 were taken into consideration. The proposed culture media thus contained the respiration-promoting Na-thioglycolate and thioctic acid as oxidizable substrates for energy generation in appropriate inorganic salts dissolved in the filtrates containing mycobactin–exochelin.

To each litre of the filtrates (IE, PE, IEM, PEM) respectively was added $(NH_4)_2SO_4$ 2 g, Na-thioglycolate 1 g, $MgSO_4$ 0·1 g, thioctic acid 0·1 g, and ferric ammonium citrate 0·05 g.

The solutions were then adjusted to pH 5·8 with KH_2PO_4. In each of the 25-ml screw-cap tubes, 12-ml aliquots of the media were distributed and sterilized for 30

Table 3. Schematic presentation: growth of mycobacteria from *M. leprae*-infected tissues in multifactorial media enriched with factors from leprosy-derived mycobacteria (LDM)

Medium: Souton: S Basal: B	Factor Donors: LDM	Factors in media		Growth in cultures	
		Exochelin	Mycobactin	Primary	Subcultures
S: IE	*M. intracell*	+		Limited 3/4	No
S: IEM		+	+	Positive 3/4	Positive 3/4
S: PE	*M.*	+		Limited 4/4	No
S: PEM	*Phlei*	+	+	Positive 3/4	Positive 3/4

min in an autoclave. The obtained media were designated as S:IE, S:IEM, S:PE and S:PEM, thus indicating their composition on Table 3.

CULTIVATION AND ESTIMATION OF GROWTH

Enrichment and adaptation

One millilitre of the host-grown *M. leprae* suspension was transferred into each of the tubes containing 20-ml media. A one-week incubation period served to adapt the host-grown cells to the *in vitro* conditions and substrates of the medium.

Primary cultures

The 7th day, cultures were shaken for 5–10 s with a Vortex Junior apparatus and transferred 1:10 into the homologue media. The cultures were incubated at 34°C.

Quantitative estimation of growth was not feasible in these primary cultures, due to the uneven clumping of the bacilli. Increased turbidity did not necessarily reflect cell multiplication, due to co-precipitation and agglomeration of bacilli with host components.

Subcultures

When microscopic examination showed a considerable increase of bacterial masses, the cultures were again transferred 1:10 into the homologue medium. This was usually effected from 4- to 6-week-old primary cultures into the subcultures.

Growth kinetics were estimated in the subcultures. Increased turbidity of the cultures was registered by nephelometric measurements, when preliminary tests indicated that cell concentration and turbidity were of linear relationship during the exponential growth phase in the subcultures. Growth kinetics as expressed in changes of nephelometric readings are only relative values, not necessarily reflecting the real rate of multiplication. This is due to the nature of the media, containing the water-insoluble mycobactins in a labile colloidal system, thus interfering with the quantum of measurable transmitted light in the optical system.

Cultures were transferred regularly at 8- to 12-week intervals.

Microscopic examination

One 4-mm loopful of the cultures was gently spread on a 1-cm diameter field on siliconized slides and dried at room temperature for 24 hr. This technique permitted adhesion to the slides during staining and the preservation of growth morphology (cords, spirals, clumps) during growth. Following treatment with

10% fresh periodic acid at 80° for 2 min regular Ziehl–Neelsen staining was performed.

Pyridine extractability of the *in vitro*-grown cells and of the bacilli recovered from the footpads was tested according to Fisher & Barksdale.[3]

Mouse footpad inoculation

Twenty Swiss albino mice were each inoculated with 10^4 acid-fast bacilli in the left hind footpad. Four mice were killed at 50-day intervals and the number of bacilli per footpad was registered.

IN VITRO DRUG SENSITIVITY OF LDM AND *M. INTRACELLULARE (LUFU)*

Three strains of LDM and *M. Lufu* were tested for drug sensitivity on Middlebrooke and Cohn's 7H10 medium. Drugs were incorporated into the media before solidification. Colonies were counted after 21 days of incubation. Results are expressed as resistance (R) or sensitivity (S) to the concentration of drugs recorded in Table 4.

The three strains, cultivated in S:IEM media from *M. leprae*-infected armadillos, were tested for drug sensitivity in S:IEM liquid media. Resistance (R) or sensitivity (S) was measured nephelometrically after 60 days of incubation.

Table 4. Schematic presentation: effects of drugs on *M. leprae* in mice and man, on leprosy derived mycobacteria and *M. intracellulare lufu*

	SM	INH	PAS	TBI	EMB	CY	ETH	DDS	CFZ	RFP
M. leprae/mice	S	S	S	S/R	R	S/R	S	S	S	S
man	R	R	R	R		R		S	S	S
(*Experientia* **34:** 1322, 1978)										
In vitro mcg/ml	10	5	10	4	16	40	40	25	20	1
M. intracell. (LDM)	R	R	R	R	R	R	S	S	S	S
M. scroful. (LDM)	R	R	R	R	R	R	S	S	S	S
M. intracell. (Lufu)	R	R	R	S/R	R	R	S	S	S	S
M. leprae? In B:IEM	R	R	R	R	R	R	R	S	S	S

SM, streptomycin; INH, isonicotinic acid hydrazide; PAS, para-aminosalicylic acid; TBI, 4-acetemino benzaldehyde thiosemicarbazone; EMB, ethambutol; CY, cycloserine; ETH, ethionamide; DDS, 4-4-diaminodiphenylsulfone; CFZ, clofazimine; RFP, rifampicin. S, sensitive; R, resistant.

Results

Multiplication of acid-fast cells was not investigated during shipment, the period of enrichment and adaptation, or in the primary cultures. These time-consuming measurements were neglected, because only cultivation in successive subcultures can be considered successful *in vitro* growth. Results in Tables 3 and 4 show that this was indeed achieved in the multifactorial media containing exochelins and mycobactins produced by any of the two LDM: *M. intracellulare* or *M. phlei*.

Four suspensions of host-grown *M. leprae* cells from armadillos were inoculated into the media shown in Table 3. Out of the four specimens, positive cultures were obtained in three trials. The three cultures had similar growth characteristics; only the latency period of growth was different, probably due to differences in the number of viable units in the inocula. For this reason cultivation of only one of the three strains will be described as a representative culture out of the three now maintained in subcultures.

In the primary cultures, there was seemingly a considerable increase in turbidity in 2–4 weeks, as well as an increase in bacterial mass microscopically. Without quantification, however, this was not considered as growth. Following 4 weeks of incubation, the cultures were transferred into the homologue media. Immediately the base line of turbidity and microscopic characteristics were registered.

The mycobactin-dependent strain of *M. paratuberculosis* grows abundantly in the Sauton medium enriched with exochelin and mycobactin. This is evidence that the media indeed contained the iron transporting factors.

Multiplication of mycobacteria in the media inoculated with *M. leprae* are shown schematically in Table 3 and semiquantitatively in Table 5. Growth was

Table 5. Nephelometric readings of the fourth subculture of mycobacteria grown in multifactorial media inoculated with *M. leprae* (armadillo)

			Nephelometric values incubation time in days				
Cultures	Media	Opalescence	0	10	30	60	80
None	S: PE	–	12			16	
	S: PEM	++++	36			46	
M. leprae	S: PE		28			32	
	S: PEM		58			186	

Heat-killed *M. leprae*. No changes in nephelometric readings. *M. paratuberculosis*. Positive cultures in the PEM media.

not observed in any of the multifactorial media if enriched with exochelins alone. However, positive cultures were obtained if media were enriched with both factors: exochelin plus mycobactin. Both LDM provided active growth-promoting exochelins and mycobactins.

Nephelometric measurements and microscopic examination left no doubt that there was a definite multiplication in the first and the following subcultures. The estimated latency period of growth is about 10–16 days, and the division time during the exponential phase is probably close to 8–12 days.

Data in Table 5 are presented as evidence of growth in the fourth subculture of one of the strains in the various media. The multiplication of acid-fast bacilli in the media was so obvious that counting of bacilli was not necessary. Turbidity measurements were made nephelometrically to visualize growth in the *M. leprae*-inoculated multifactorial media.

Growth occurred as a flocculant sediment which was easily, but not completely, homogenized by shaking for 5 sec with the Vortex apparatus. Culture smears showed a tendency of the cells to clump together. When sedimented for 1 min on siliconized slides, it became evident that the flocculant growth consisted of small, and often extremely large, clumps or cordlike arrangements of the relatively large acid-fast rods. Acid fastness of the bacilli was extracted by pyridine.

DRUG SENSITIVITY OF LDM

Data presented in Table 4 show that, while all the tested antituberculous drugs except ethambutol have a complete or partial suppressing effect on *M. leprae* in the footpads of mice, only DDS and clofazimine have a slow and rifampicin a fast and potent therapeutic effect on leprosy in man.

Results show that only those drugs which show established clinical efficiency in human leprosy have inhibiting effects on the growth of LDM and *M. intracellulare (Lufu)*.

Rifampicin, the most potent drug in human leprosy, with a minimal bactericidal concentration of 1 to 1 μg ml in the serum of mice, inhibited completely the growth of our cultures in a dose as low as 1 μg ml. A relatively high concentration of 25 μg ml of DDS and 20 μg clofazimine was necessary to inhibit the growth of the same cultures *in vitro*. The minimum inhibitory concentration of DDS is 0·01–0·03 μg ml in the serum of mice against *M. leprae* in the footpad. DDS, however, is a slow-acting drug in man and has only bacteriostatic effects. All the strains isolated from human and armadillo leprous tissues showed resistance against all the other anti-tuberculous drugs tested.

Animal inoculations

The fourth subculture grown in PEM medium was inoculated into the footpads of

mice. The growth of the bacilli in the mouse footpad was somewhat faster than the usual pattern obtained following injection of host-grown *M. leprae* (probably due to the presence of mycobactin). When 10^4 acid-fast bacilli were injected per footpad, an average of 2×10^6 cells were recovered in 160 days. Bacilli recovered from the footpads lost their acid fastness after pyridine extraction, but remained Gram positive.

Discussion

In addition to previously published data,[9–13] the following experimental evidence is offered as indicative that *M. leprae* might indeed be a microbe-dependent microorganism and that the obtained cultures are probably identical to *M. leprae* or cultures mixed with LDM.

Media were inoculated with authentic host-grown *M. leprae* cells.

Cells grow in special multifactorial media.

Growth occurred under physical conditions optimal for the endogenous respiration of *M. leprae*: pH 5·8 at 34°C.[8]

Both known respiratory stimulants of *M. leprae*,[8] a SH compound, Na thioglycolate, and a yeast extract growth factor, thioctic acid,[1,21] were efficient supplements in media in which growth was obtained.

Positive cultures were obtained in media containing both exochelins and mycobactin[15] supplied by a LDM: *M. phlei*.

Cultures did not grow on Löwenstein or in Dubos media, but in the footpads of mice produced the disease similar to that caused by host-grown *M. leprae*.

Strong acid fastness of the cells was eliminated by pyridine.

Cultures show a resistance pattern to antibacterial agents, comparable to drug sensitivity of *M. leprae* in man.

The frequent presence of LDM in *M. leprae*-infected tissues is well documented. LDM are hard to grow and are present in extremely small numbers in leprotic tissues. It is highly probable that more leprosy infected hosts harbour LDM than are reported in the literature. Little attention was paid to the possibility that LDM were present at anatomical locations remote from the *M. leprae*-infected sites. It is known that secondary strains of mycobacteria were cultivable from organs remote from the tissues infected with *M. paratuberculosis* in Johne's disease of cattle. This microbe-dependent microorganism is dependent on growth factors of the secondary mycobacteria from a remote organ. A similar mechanism in leprosy remains to be investigated.

It was previously proposed[10] that attempts to cultivate *M. leprae* must be focused on media highly selective for *M. leprae*, without promoting the growth of the accompanying cultivable mycobacteria. This problem is complicated by the fact that *M. leprae* is the slowest of the slow growers and even the slow-growing secondary species will overgrow *M. leprae* in nonselective media. It is predictable

that *M. leprae* might also be grown and isolated from nonselective semi-solid media or, when the secondary LDM are present, at sites remote from the *M. leprae*-infected lesion of which cultivation is attempted.

It did not become evident whether cultures were pure or of a mixed nature.

The presented results support the view of Hall *et al.*[5] that to grow '*M. leprae* is probably a multifactorial problem'. In a multifactorial medium several components might be replaced, but exochelins and mycobactin are a *sine qua non* of success. The findings of Macham *et al.*[15] were of practical importance in obtaining exochelin and mycobactin-enriched media using Tween 80 containing spent cultures.

The *in vitro* drug sensitivity of LDM merits special attention. Since the *in vitro* cultivation of *M. leprae* remains to be proven, no *in vitro* pharmacological model is available for screening substances with prospective antileprosy effect. The only reliable test subject for screening drugs for activity against leprosy is the human lepromatous leprosy patient. LDM, however, presents a characteristic resistance pattern to antibacterial agents that is comparable to the drug sensitivity of *M. leprae* in man. This knowledge leads to the proposal of an *in vitro* method using LDM for screening drugs against leprosy.

Results presented in this communication have furnished further indirect but strong experimental evidence that *M. leprae* might be a microbe-dependent microorganism. This concept is supported by the evidence that multiplication of mycobacteria occurred only in *M. leprae*-inoculated multifactorial media which contained growth factors produced by LDM. The question thus arises as to whether antileprosy therapy should be targeted against *M. leprae* or against the donors of growth factors: the LDM. The results presented clearly show that LDM are highly sensitive to drugs which show therapeutical effects in lepromatous leprosy patients. I propose that the curative effects of antileprosy agents act by eliminating the secondary mycobacteria (LDM) which seem to be an integral part of the pathology as etiological cofactors in leprosy.

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