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In Vitro Cultivation of *Mycobacterium X* from *Mycobacterium leprae*-infected Tissues in Acetone-dimethylsulfoxide-tetradecane Medium[1]

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It was previously reported ([12, 13, 14]) that a hitherto unidentified *Mycobacterium X* was regularly cultivable from *M. leprae*-infected tissues from humans and armadillos in dimethylsulfoxide (DMSO)-tetradecane medium. The strains were strongly acid fast, did not grow on Löwenstein-Jensen or in Dubos media. The tested cultures produced the disease characteristically obtained following the injection of *M. leprae* into the foot pads of mice. Cultivation of *Mycobacterium X* in the two-phase system medium, however, had several disadvantages. The growth was extremely slow. Estimation of growth was difficult to quantitate and separation of the bacilli from the medium was difficult. Attempts were therefore made to replace or emulsify the oily tetradecane in the medium. Since the terminal methyl bindings of the straight chain hydrocarbons (and probably DMSO) are known carbon and energy sources for growth of most species of mycobacteria ([2, 4, 6, 7, 10, 21, 22]), other water soluble straight chain chemical configurations with terminal methyl bindings were obvious candidates to replace tetradecane. Dimethylketone (acetone) was the ideal substance to be tested, because it is freely miscible with water and acetone is also a solvent for tetradecane. Acetone is a minute constituent of normal animal tissue and present in high concentration in tissues during malnutrition and diabetic ketosis. The chemical structure of acetone, resembling DMSO and tetradecane, is such that the terminal methyl bindings of acetone might serve as reactive sites for enzymatic oxidation and consequent energy generation.

Cultivation of *Mycobacterium X* in a chemically well-defined medium, containing acetone, DMSO and tetradecane will be reported in this communication.

MATERIALS AND METHODS

Sources of *M. leprae*. Four human lepromata were received from Brazil (H1, H2, H3, H4), two from India (H5, H6) and six armadillo spleen specimens (A6, A7, A8, A9, A10, A11) were received from Melbourne, Florida, U.S.A. The human tissue specimens were transported without refrigeration in the basal medium. Human specimens arrived one to three weeks following biopsy. One of the armadillo spleen specimens was inoculated into the media within a few hours after autopsy. Five spleen specimens were kept frozen at −20°C for two to four months before shipment to our laboratory. The bacilli were separated by partial purification as described below and then shipped to Montreal, Canada, in the basal solution. The suspensions were en route for three to seven days. The specimens were washed with distilled water and cut with scissors into small pieces. These were homogenized in a Potter-Elvehjem homogenizer to obtain a 10% (w/v) suspension in the basal solution. The suspensions were diluted with equal volumes of 4% NaOH solution and kept for 25 min at 37°C. The suspension was centrifuged for 10 min at 6000 rpm. The sediment was washed once with the basal solution and centrifuged at 1000 rpm for 1 min. The sediment obtained was discarded and the supernatant was centrifuged for 10 min at 6000 rpm.

Inoculation of the media. The sediment was diluted in the medium without tetradecane, to contain 2×10^8 acid-fast rods per 1 ml suspension; 0.5 ml was added to each of the tubes containing 10 ml of the complete liquid medium. Counting of the

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acid-fast rods was feasible only in the absence of the oily tetradecane.

Acetone-DMSO-tetradecane medium. In one liter of distilled water the basal medium contained KH_2PO_4 7.0 g, Na_2HPO_4 1.0 g, $(NH_4)_2SO_4$ 2 g, iron-ammonium citrate 0.1 g, $MgSO_4$ 0.1 g and dimethylsulfoxide (DMSO) 10 ml. The pH was 6.0. The basal medium was autoclaved for 30 min. After cooling to room temperature, 150 ml of acetone (U.S.P. grade) was added to 850 ml of the basal medium. Ten ml of the basal medium, supplemented with acetone, was distributed into each 50 ml sterile screw cap tube.

Tetradecane was sterilized by autoclaving for 30 min. To each tube containing 10 ml sterile acetone-DMSO medium, 0.1 ml sterile tetradecane was added aseptically.

All containers, pipettes, centrifuge tubes and syringes were made of glass, since acetone and DMSO react with plastic materials.

Incubation and estimation of growth. The cultures were incubated at 38°C. They were shaken with a Vortex homogenizer twice weekly. All cultures were observed during at least two months of incubation.

Because of the uneven distribution of bacilli in the cultures, exact counting of bacilli was not feasible in the liquid medium. Turbidity measurements with simultaneous microscopic examinations were therefore used for the quantitative evaluation of growth during the incubations. A Klett photoelectric colorimeter, with a 540 μm filter, and 10 mm diameter standard tubes were used. Cultures were shaken vigorously for 10 sec with a Vortex homogenizer immediately prior to taking the Klett unit measurements.

The cultures were simultaneously examined in microscopical preparations. Immediately following mechanical shaking (Vortex), a 3 mm loopful from the cultures was spread evenly on a 2 cm diameter surface of a silicone-coated slide. The smears on the slides were allowed to dry 20–24 hr. Fixation was achieved over flame from a gas burner. A freshly prepared 5%–10% solution of periodic acid was poured on the slide and warmed gently for 2 min, without boiling. After washing in a tap water bath, the preparation was stained by the Ziehl-Neelsen method.

Silicone-coated slides. Cells were easily washed off glass slides during the staining procedure. Therefore, the glass slides had to be coated by dipping them in a 0.1% solution of silicone in chloroform. The coated slides were dried at 60°C in an oven overnight, followed by heating at 250°C for 1 hr in a hot air oven.

RESULTS

The turbidity of the cultures did not increase over a two-month period of observation in the acetone-DMSO-tetradecane media inoculated from the specimens of human origin marked H2 and H4. A poor quality inoculum was noted microscopically, showing only a few solidly stained acid-fast rods after 24 hr of incubation, and the morphology of the acid-fast rods deteriorated progressively.

Within five to 12 days there was a slight but visible increase in the turbidity of the media which were inoculated from specimens H1, H3, H5, H6, as well as A6, A7, A8, A9, A10 and A11. The growth became visible as a fine white emulsion floating on the surface of the medium. Following vigorous mechanical shaking by the Vortex apparatus, the growth became quite homogenous, but became diphasic again within a few hours, as seen by the separation of the oily tetradecane on the surface of the medium.

The turbidity of the Vortex-homogenized cultures increased gradually with time and reached a peak within six to 12 weeks as seen by the Klett unit measurements. Microscopic examination showed that the increased turbidity was due to the multiplication of bacilli rather than to chemical or physical changes in the media. This was also strengthened by the fact that noninoculated controls remained clear of any precipitate and that media inoculated with heat-killed bacilli showed no significant changes in turbidity.

Microscopic examination showed bacilli in the growing cultures to be strongly acid fast, appearing mostly as single rods or attached side by side or end to end. Some small, large, or extremely large but loose clumps were also seen microscopically.

THE TABLE. *Increase of turbidity (in Klett units) of the fourth subcultures of* Mycobacterium X *in the acetone-DMSO-tetradecane media during 80 days of incubation.*

In vitro grown *Mycobacterium X*	Incubation time in days					
	0	10	20	30	60	80
A6	26	22	48	102	320	314
A8	14	18	35	56	208	254
A10	18	24	52	98	296	336
Medium alone	6	8	10	10	14	12
A8 heat-killed	22	16	14	20	22	20

Acid-fast rods were often very long; most of them were of equal length. Beaded forms and branching were seldom seen. Proportionately with the increases in turbidity of the cultures, the bacterial masses, as well as the number and size of the acid-fast clumps, increased steadily. This seemed to be a clear indication that the increases in turbidity were due to multiplication of cells rather than liberation of bacilli from disintegrating minute tissue fragments.

It was not possible to establish the lag period, the logarithmic growth or the plateau, since continuous density measurements were not taken. For this reason subculturing was arbitrarily attempted when the turbidity increased above 250 to 300 Klett units and microscopic examination showed considerable increase in bacterial mass. An increase in Klett unit readings alone in the primary cultures was not accepted as evidence of cell multiplication, since changes in tissue fragment size might interfere with the turbidity of the cultures. These results, therefore, are not presented for the primary cultures, although microscopic examinations left no doubt that considerable increases of bacterial mass occurred.

Subcultures. At the end of the second month of the active growth phase, the cultures were transferred (1:10) into fresh acetone-DMSO-tetradecane media. The growth pattern and growth characteristics in the subcultures were the same as in the primary cultures. Counting of bacilli was again technically difficult because of the emulsion type growth which had to be estimated by Klett unit measurements and by microscopic examination. The lag period in the subcultures was more regular, about ten to 14 days. Further transfers were made at two-month intervals. From the total of 10 experiments, 5 armadillo and 2 human strains are now maintained and growing regularly as subcultures in the liquid media. The Table shows quantitative evidence of multiplication in the fourth subculture as estimated at different time intervals after inoculating three of the five armadillo strains into the acetone-DMSO-tetradecane liquid medium. This is presented as examples and prototypes of growth in the subcultures.

Again microscopic examination revealed that changes in turbidity were due to proportional increases in bacterial mass. Furthermore, the carryover of tissue fragments into the fourth subculture was negligible, thus not interfering with turbidity measurements.

The cultures were tentatively designated as *Mycobacterium X*. The cultures did not grow on Löwenstein-Jensen or in Dubos liquid media during three months of incubation at 38°C.

Animal inoculation. Three of the cultures obtained from *M. leprae*-infected armadillos were injected into the foot pads of mice. Bacilli were siphoned from the surface of the fourth subculture. The fine emulsion was diluted with 20% acetone in water to obtain 5×10^3 acid-fast bacilli per 0.05 ml. Using a tuberculin syringe, this amount was injected from each culture respectively into the hind foot pads of ten Swiss albino mice. The mice were killed five months later. The number of acid-fast rods were counted in the pooled homogenized foot pad connective tissues of ten mice. Depending on the strains, the number of acid-fast bacilli increased from 5×10^3 to $1.2–2.2 \times 10^5$ cells.

DISCUSSION

The results presented in this communication show that hitherto unclassified and still ill-defined strains of mycobacteria can be cultivated from human and armadillo

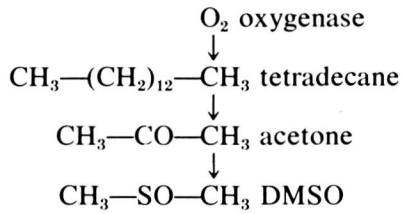
leprosy tissues in media containing short chain compounds with two terminal methyl bindings: dimethylketone (acetone), dimethylsulfoxide (DMSO) and tetradecane. The cultures are preliminarily designated as *Mycobacterium X*.

Cultures and subcultures with identical growth characteristics were obtained from a high proportion of human and armadillo leprosy specimens from different geographical locations. A special medium and special physical conditions were required to obtain growth. *Mycobacterium X* did not grow on media which support the growth of other species of mycobacteria. The three strains tested so far multiply in the foot pads of mice in a fashion which is similar to the multiplication occurring after injection of *M. leprae*. *Mycobacterium X* is a very slow-growing mycobacterium *in vitro* and multiplies in the mouse foot pad at a rate comparable to that of *M. leprae*. The rate is not necessarily identical to that of *M. leprae*, since there was only one harvest after five months and it is conceivable that the 10^5 level was reached earlier.

The growth conditions for *Mycobacterium X* are not conventional, but neither are they unusual, as suggested by the following evidence:

1. Straight chain hydrocarbons are preferred sources of carbon and energy for most species of mycobacteria [2, 4, 6, 7, 10, 21].
2. Terminal methyl bindings are successively oxidized into the corresponding primary alcohol, monoic acid, hydroxymonoic acid and dioic acid by several species of microorganisms [1, 3, 5, 8, 17, 18, 19].
3. Mycobacteria utilizing hydrocarbon will also utilize acetone as the sole source of carbon and energy [19].
4. Acetone is present in animal tissues in physiological and pathological conditions. Consequently this (or other ketones) might be *in vivo* carbon and/or energy sources for *Mycobacterium X*.

DMSO also furthered multiplication of mycobacteria considered to be *M. leprae* in the experiments of Olitzki, *et al.* [20] and in the experiments of Kato [12, 13, 14]. The terminal methyl groups of DMSO may be the targets of enzymatic oxidation and metabolization by *M. leprae*. The *n*-alkanes (such as tetradecane), as well as acetone and DMSO, might be enzymatically oxidized at one of the methyl groups by the same enzyme or, alternatively, the three substrates with terminal methyl groups might require different enzymes for oxygenation.



Kato, *et al.* [15] have suggested that *M. leprae* have certain autotrophic characteristics similar to some hydrogen metabolizing bacteria [19]. If an autotrophic system is really functional as a biosynthetic process, leprosy bacilli should be competent enough to grow on nutrients as simple as acetone, DMSO and tetradecane.

Because of the structural similarity of acetone and DMSO to the straight chain hydrocarbons and because *Mycobacterium X* was first cultivated in media supplemented with tetradecane, the *in vitro* nutritional role of acetone and DMSO can be explained in the light of results obtained with straight chain hydrocarbons in the media for cultivation of microorganisms in general and mycobacteria in particular [1, 2, 6, 7, 19, 21].

It was pointed out that mycobacteria which presumably had not previously been exposed to hydrocarbons could oxidize *n*-alkanes and grow on them as the sole source of carbon [4, 5, 8, 9]. Oxidation and growth occur with or without an adaptation period [1]. The affinity to hydrocarbons is a genetic property of mycobacteria independent of their origin and history of cultivation [1, 2, 6]. It can therefore be expected that leprosy bacilli might grow on hydrocarbons, whether or not they have previously been exposed to these molecules in nature or in the host.

Kallio [10] was the first to draw the attention of students of leprology to the physiological implications of hydrocarbons with regard to mycobacteria. Recently Kato and de Thököly [14] advocated that among the petroleum hydrocarbons, the medium length

chain *n*-alkanes might be ideal substrates for mycobacteria as energy sources and proposed cultivation trials for *M. leprae* in a liquid medium containing an *n*-alkane.

A high yield of biomass occurs whenever cells are grown on hydrocarbons. These substantial yields of microbial cells result from the high energy content of the hydrocarbons and partly from the efficient carbon fixation by the cells from the hydrocarbon substrates ([3, 5, 8]). Microorganisms which oxidize hydrocarbons can consume oxygen at an unusually fast rate ([1]). Microbial strains either have the capacity to or can adapt within minutes to oxidize hydrocarbons ([5, 17, 18]).

Acetone (dimethylketone), DMSO, and tetradecane were the prospective carbon and energy sources in the present medium. The chemical configuration of acetone and DMSO bears a resemblance to tetradecane, previously used in the media for *Mycobacterium X* ([11, 13, 14]). Enzymes which oxidize the α and ω methyl groups in tetradecane might recognize the terminal methyl groups in acetone and DMSO as reaction sites for enzymatic oxidation.

Acetone and DMSO in the proposed medium have several advantages. They are small molecules with terminal methyl bindings. Both are water miscible and fat soluble, permitting easy penetration through membranes. Acetone is a by-product of biological reactions in the tissues during starvation, inappropriate diet, and diabetes. Admittedly, acetone is an unusual constituent of a culture medium for the orthodox microbiologist, but because of the physiological and pathological presence of acetone in the tissues, as a consequence of fatty acid degradation in the cells, this ketone merits further attention as a supplement in culture media.

The simultaneous presence of the three compounds—acetone, DMSO, and tetradecane—was necessary to obtain reproducible *in vitro* growth of *Mycobacterium X*. Two of these—tetradecane and DMSO—were necessary in previous experiments for the *in vitro* cultivation of *Mycobacterium X* from leprosy-derived tissues ([12, 13, 14]). The present results do not explain this multiple requirement of *Mycobacterium X* for the three compounds. Acetone and/or DMSO could facilitate tetradecane uptake as solvents or emulsifiers without contributing as nutrients per se to multiplication of the bacilli in the medium.

The relationship of *Mycobacterium X* to the pathology of leprosy is not yet clear. However, this is the first time that slow-growing strains of mycobacteria, which do not grow on Löwenstein or in Dubos media, are reported to grow on a special medium in primary cultures and in subcultures and to multiply in the foot pads of mice in a fashion similar to that of *M. leprae*.

SUMMARY

Several strains of mycobacteria were cultivable from *Mycobacterium leprae*-infected human and armadillo tissues in a liquid medium containing three dimethyl analogs: dimethylketone, dimethylsulfoxide, and tetradecane [$CH_3 \cdot (CH_2)_{12} \cdot CH_3$]. The medium contained KH_2PO_4, 7.0 g; Na_2HPO_4, 1.0 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4$, 0.1 g; iron ammonium citrate, 0.1 g; DMSO, 10 ml; and acetone, 150 ml in distilled water *ad* one liter. Tetradecane 0.1 ml was added aseptically to each tube, containing 10 ml of the sterile medium. The media, inoculated with *M. leprae*, were incubated at 38°C and shaken vigorously twice weekly.

Growth developed as a fine emulsion at the upper phase of the two-phase system. This was homogenized by mechanical shaking, permitting growth estimation by turbidity measurements. Microscopic examination showed unmistakably the slow but abundant multiplication of acid-fast rods.

The logarithmic growth rate was measurable during two to three months, followed by a plateau. The strains are maintained in subcultures by regular transfer into the same medium at two- to three-month intervals. The cultures and subcultures do not grow on Löwenstein or in Dubos media, but in the foot pads of mice they produce a multiplication similar to that obtained following injection of host-grown *M. leprae*. The cultures are tentatively designated as *Mycobacterium X*. The relationship of *Mycobacterium X* to the pathology of leprosy is not clear.

RESUMEN

Se cultivaron varias cepas de micobacterias aisladas de tejidos humanos y armadillos infectados con *Mycobacterium leprae* en un medio líquido conteniendo tres dimetil-análogos: dimetil-cetona, dimetil-sulfóxido y tetradecano [$CH_3 \cdot (CH_2)_{12} \cdot CH_3$]. El medio con-

tenía 7.0 g de KH_2PO_4; 1.0 g de Na_2HPO_4; 2.0 g de $(NH_4)_2SO_4$; 0.1 g de $MgSO_4$; 0.1 g de citrato férrico amónico; 10 ml de DMSO; 150 ml de acetona y agua destilada hasta 1000 ml. A cada tubo conteniendo 10 ml del medio estéril se adicionó, en forma aséptica, 0.1 ml de tetradecano. Los medios inoculados con *M. leprae* se incubaron a 38°C y se agitaron enérgicamente dos veces por semana.

El crecimiento se desarrolló como una emulsión fina en la fase superior del sistema de dos fases. La emulsión se homogeneizó por agitación mecánica y el crecimiento se cuantificó por turbidimetría. El examen microscópico mostró la lenta pero abundante multiplicación de bacilos ácido-resistentes.

Durante los siguientes 2 a 3 meses de iniciado el cultivo el crecimiento fue logarítmico, después alcanzó su meseta. Las cepas se mantienen en subcultivos por transferencia en el mismo medio a interválos de 2 ó 3 meses. Los cultivos y los subcultivos no crecen en medios de Löwenstein o Dubos pero en los cojinetes plantares del ratón producen una multiplicación similar a la obtenida por inyección del *M. leprae* aislado de humanos. Los cutivos han sido tentativamento denominados como *Mycobacterium X*. La relación del *Mycobacterium X* con la patologia de la lepra no es clara.

RÉSUMÉ

On a pu cultiver plusieurs souches de mycobactéries obtenues à partir de tissus humains et tatous infectés par *Mycobacterium leprae,* dans un milieu liquide contenant trois analogues dimethylés: dimethylcetone, dimethylsulfoxyde, et tetradecane [$CH_3 \cdot (CH_2)_{12} \cdot CH_3$]. Le milieu contenait du KH_2PO_4, à raison de 7.0 g; Na_2HPO_4, à raison de 1.0 g; $(NH_4)_2SO_4$, à raison de 2.0 g; $MgSO_4$ à raison de 0.1 g; du citrate ammonique de fer à raison de 0.1 g; du DMSO à raison de 10 ml; et de l'acétone à raison de 150 ml dans de l'eau distillée pour faire un litre. A chaque tube, contenant 10 ml du milieu stérile, on a ajouté de manière aseptique 0.1 ml de tetradecane. Les milieux inoculés avec *M. leprae* ont été incubés à 38° C et secoués vigoureusement deux fois par semaine.

La croissance s'est présentée sous forme d'une émulsion fine à la phase supérieure d'un système à deux phases. Cette émulsion a été homogénéisée par vibrations mécaniques, ce qui a permis une estimation de la croissance par des mesures de turbidité. L'examen microscopique a montré de manière indéniable qu'il se produisait une multiplication lente mais abondante de bâtonnets acido-résistants.

La croissance logarithmique pouvait être mesurée pendant deux à trois mois; elle était suivie par un plateau. Les souches ont été maintenues en sous-cultures par des passages réguliers dans le même milieu, à des intervalles de deux à trois mois. Les cultures et les sous-cultures ne poussaient pas sur les milieux de Löwenstein ou de Dubos, mais dans les coussinets plantaires de la souris elles produisaient une multiplication similaire à celle que l'on obtient à la suite de l'injection de *M. leprae* recueillie chez un hôte. Les cultures ont été désignées provisoirement sous le terme de *Mycobacterium X*. La relation du *Mycobacterium X* avec la pathologie de la lèpre n'est pas claire.

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