asites" or "noncultivable mycobacteria" or "metabolically deficient" organisms? Certainly a great many highly qualified scientists have devoted years of their lives to pursuing this elusive goal without success. Do younger researchers feel that their careers will be threatened if they do not produce fast results? Are the controversies which have surrounded previous claims of cultivation of *M. leprae* discouraging younger investigators from taking up the task? Responsible persons and agencies must find a way to encourage research on the cultivation of *M. leprae*. What an exciting and rewarding field this is, to force that elusive microorganism to split in a test tube! Millions would benefit in a not-too-distant future from the discovery. Are we guilty of scientific negligence if we fail to continue to emphasize this goal?

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Isolation of Fastidiously Growing Mycobacteria from Armadillo Livers Infected with *Mycobacterium leprae*

To the Editor:

In 1980 we received four armadillo livers infected with *Mycobacterium leprae*. Their origin is indicated in Table 1. The specimens were transported on dry ice and kept in the laboratory in an electric deep freeze at −70°C until use.

Specimen I was from an animal with a "natural" *M. leprae* infection ([18]), while the other three were from animals injected with *M. leprae* extracted from human biopsy material.

In our laboratory each liver was aseptically divided into different samples. Suspensions in distilled water (50% w/v) were prepared and investigated on different dates (Table 1). The viability of the acid-fast bacilli (AFB) was tested by titrations in mouse foot pads (MFP), starting with an inoculum of 5×10^3 per foot pad. Animals were examined tri-monthly from the sixth month on, and five of them killed and counts performed when 5×10^5 AFB/MFP was reached; the last examinations were performed one year after inoculation.

Two-tenths ml amounts of the same suspensions were inoculated on the surface of slants of modified Ogawa medium ([10]) either without prior treatment or after addition of different concentrations of hydrochloric acid or sodium hydroxide (final concentrations from 0.1% to 2% and neutralized or not). Modified Ogawa medium was prepared by the addition of autoclaved suspensions of one of the following mycobacterial species: *M. phlei*, *M. lufu* ([12]), *M. lepraemurium* ([10]) or *M. leprae* (from human biopsies or armadillo livers) and adjusted to different pH values ([15]). Incubation was at 30°, 33°, and 37°C, with or without CO_2 and in incubators, humidified or not, stoppers pierced with 19G needles or not ([10, 14]), for a period of one year.

Results of titrations in MFP revealed that

TABLE 1. *Origin of the infected armadillo livers, date of investigation, and number of acid-fast bacilli (AFB) per ml suspension.*

Liver no.	Origin	Armadillo no.	Sample no.	Date of investigation	AFB/ml
I	A.F.I.P.[a], Washington (Dr. G. P. Walsh)	2010 Natural infection	A1	5/13/81	2.68×10^8
			A2	5/20/81	1.50×10^8
II	A.F.I.P.[a], Washington (Dr. G. P. Walsh)	6 Experimental infection	A3	6/ 2/81	1.00×10^9
			A4	6/11/81	1.02×10^9
			A11	11/24/81	6.50×10^9
III	I.P.[b], Cayenne (Dr. Y. Robin)	AJ Experimental infection	A5	6/23/81	6.22×10^7
			A10	11/17/81	2.30×10^7
IV	N.I.M.R.[c], London (Dr. R. J. W. Rees)	2457/10 Experimental infection	A7	7/28/81	3.00×10^8
			A8	9/24/81	5.00×10^8

[a] Armed Forces Institute of Pathology.
[b] Institut Pasteur.
[c] National Institute for Medical Research.

liver I contained 0.1% multiplying AFB, liver II less than 0.01%, and liver III 30%. Results for liver IV are not yet available.

No *in vitro* cultures were obtained from livers I and II. Colonies developed after 1 to 6 months' incubation on media inoculated with livers III and IV. Percentages of positive tubes varied between 2.7% and 27.3%, but were always higher from samples pretreated with HCl or NaOH than without pretreatment. Growth appeared only on Ogawa medium at pH 5.5 to 6.0 containing suspensions of autoclaved *M. lufu* ([12]), *M. lepraemurium* or *M. leprae*. The number of colonies per tube varied between 10 and 100. Löwenstein-Jensen (LJ) media always remained negative. Subcultures were performed on Ogawa and LJ. There was no growth on LJ when the inoculum contained less than 10^6 AFB. On Ogawa medium, one colony forming unit (CFU) corresponds to 10^5 AFB. The strains grow better at 37°C than at 33°C; a humidified CO_2 enriched atmosphere is not beneficial.

These strains of armadillo-derived mycobacteria (ADM) were identified by the tests previously described ([5,9]), using inocula containing at least 10^9 AFB. Three slightly different scotochromogenic strains may provisionally be recognized, as shown by the characters of six representative strains in Table 2. Strains 8480 and 8489 resemble *M. scrofulaceum*; 8251 and 8263 resemble *M. gordonae*; 8233 and 8335 may be related to the MAIS complex ([11]) although all differ from the type species, being sensitive for isoniazid and hydroxylamine. Since the growth range at different pH values was recently found to be an important taxonomic character within the genus *Mycobacterium* ([15]), this was also studied with the ADM. Strain 8480 has the same optimal pH growth range as *M. scrofulaceum*, between 5.4 and 6.5. For the *M. gordonae*-related and MAIS complex-related strains the pH was extremely critical, growth optimum being limited between pH 5.4 and 5.7.

During the present work, cultivation attempts with 98 human biopsies and 127 mouse foot pad harvests were performed in the same laboratory using similar although slightly different experimental conditions. Not a single colony of AFB was ever obtained. This and the fact that ADM were obtained from aliquots of livers manipulated at different times leads to the assumption that the strains obtained are not laboratory contaminants.

Our *in vitro* studies of these ADM have also shown that high numbers of AFB are needed to obtain one CFU (10^5 on Ogawa media and 10^6 on Löwenstein). These results imply that the armadillo livers III and IV were probably heavily infected with these difficult to grow mycobacteria.

Three important factors influence the *in vitro* growth of these armadillo derived mycobacteria: a) cultures develop only on media at low pH (5.4–5.7), b) large inocula are necessary, c) their isolation in primary

TABLE 2. In vitro *characteristics of cultivable mycobacteria isolated from armadillo livers.*

Strain no.		LJ[a]	Pigment	Growth at (°C) 37	42	Resistance to I	T	H	P[b]	Catalase >45 mm	Nitrate red.	Acid phosphatase	Tween hydrolysis	Urease	Putrescine	Colony on OAA[c]
Liver III	8233	−	Sc	+	−	−	+	−	+	−	−	−	−	+	−	Sm S
	8480	−	Sc	+	−	−	+	−	+	+	−	−	−	+	−	Sm S
	8251	−	Sc	+	−	−	+	−	+	+	−	−	+	+	−	Sm S
Liver IV	8335	−	Sc	+	−	−	+	−	+	−	−	−	−	+	−	Sm S
	8489	−	Sc	+	−	−	+	−	+	+	−	−	−	+	−	Sm S
	8263	−	Sc	+	−	−	+	−	+	+	−	−	+	+	−	Sm S
M. avium[d]		+	−	+	+	+	+	+	+	−	−	−	−	−	−	SmS-SmT
M. scrofulaceum[d]		+	Sc	+	+	+	+	+	+	+	−	−	−	+	−	SmS-SmK
M. gordonae[d]		+	Sc	+	−	−	+	+	+	+	−	+	+	+	−	SmS-SmK

Sc = Scotochromogenic

[a] No growth with $<10^6$ AFB.

[b] I = isoniazid 10 mg/l
T = thiophene-2-carboxylic acid hydrazide 1 mg/l
H = hydroxylamine hydrochloride 250 mg/l
P = para-nitrobenzoic acid 500 mg/l

[c] OAA = oleic acid albumin agar. For colony morphology descriptions see (9).

[d] Data from Jenkins, *et al.* (5).

culture is considerably favored by pretreatment of the organ suspensions with acid or alkali.

The same factors are also of prime importance for the *in vitro* growth of another "difficult to grow mycobacterial species," *M. lepraemurium* ([10, 13, 14]).

From our *in vitro* studies on the influence of the pH of the media, there is evidence that the ADM are able at least to survive in phagolysosomes as is the case with other intracellular parasites ([1,2,3,6,7]). This is another argument for their being present in the tissues and not being laboratory contaminants. If the ADM are tissue contaminants they must have been present in the armadillo tissues before inoculation of the animals with *M. leprae* or they were introduced simultaneously, or later on, in any case multiplying together with the inoculated *M. leprae*. Nakamura, *et al.* ([8]) and Prabhakaran, *et al.* ([17]) have also isolated scotochromogenic mycobacteria from tissues.

The precise identification of the ADM and their relationship with other mycobacteria, including *M. leprae* ([4,16]), have to be further studied, as should more tissues from uninoculated and *M. leprae*-inoculated armadillos.

The presence of ADM in such proportions may influence the results of any studies performed with armadillo-derived *M. leprae,* and all tests to detect their presence should make use of techniques similar to those used by us—a full account of which will be presented shortly—and not be limited to the inoculation of LJ or Dubos media incubated for six weeks.

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