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BACTERIOLOGICAL STUDIES OF ARMADILLO LIVERS INFECTED WITH *MYCOBACTERIUM LEPRAE*

by

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Summary — Non mycobacterial contaminants and *in vitro* cultivable mycobacteria have been isolated in large numbers from two out of four armadillo livers infected with human derived *Mycobacterium leprae*. The organisms were « difficult to grow mycobacteria » and their *in vitro* multiplication was only successful if the following three conditions were fulfilled : (1) inocula should contain high numbers of viable organisms ($> 10^5$); (2) suspensions should be pretreated with NaOH or HCl; (3) acid media, with a very precise pH (5.4-5.7) and containing autoclaved mycobacterial suspensions should be used.

The presence of these mycobacteria in armadillo livers may influence the results of studies performed on *M. leprae* purified from such organs. The relationship of these mycobacteria with *M. leprae* remains to be elucidated.

KEYWORDS : Mycobacteria, *in vitro* Growth; *Mycobacterium leprae*; Armadillo; Liver.

Introduction

With the availability of armadillo tissues containing large numbers of *Mycobacterium leprae*, new efforts can be made to cultivate this organism *in vitro*, since it can be expected that « difficult to grow mycobacteria » do so only from large inocula (Pattyn & Portaels, 1980; 1981).

During 1980, four different armadillo livers, infected with *M. leprae* were received in our laboratory and bacteriologically examined. The results are presented in this paper.

Materials and Methods

Livers

The origin of the four livers is indicated in table 1. Liver n° 1 was from an animal infected with natural armadillo leprosy (Walsh *et al.*, 1977), the others from animals injected with human derived *M. leprae*.

Transportation was on dry ice and conservation in the laboratory was in an electric deep freeze at − 70 °C. Specimens 1 and 2 were 15 g fragments divided over several vials while livers 3 and 4 arrived as whole organs.

Separate vials of livers 1 and 2 were used at different dates, as were pieces of liver from specimens 3 and 4 (table 1).

TABLE 1

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

Liver	Origin	Armadillo n°	Samples n°	Date of investigation	AFB/ml
1	A.F.I.P. (1), Washington	2010	A1	13.05.81	$2.68\ 10^8$
	(Dr. G.P. Walsh)	Natural infection	A2	20.05.81	$1.5\ 10^8$
2	A.F.I.P., Washington	6	A3	02.06.81	10^9
	(Dr. G.P. Walsh)	Experimental infection	A4	11.06.81	$1.02\ 10^9$
			A11	24.11.81	$6.5\ 10^9$
3	I.P. (2), Cayenne	AJ	A5	23.06.81	$6.22\ 10^7$
	(Dr. Y. Robin)	Experimental infection	A10	17.11.81	$2.3\ 10^7$
	N.I.M.R. (3), London	2457/10	A7	28.07.81	$3.\ 10^8$
4	(Dr. R.J.W. Rees)	Experimental infection	A8	24.09.81	$5.\ 10^8$

(1) Armed Forces Institute of Pathology.
(2) Institut Pasteur.
(3) National Institute for Medical Research.

Surface sterility testing

The surface of the organs and inside of the containers were sampled with cotton applicators and inoculated onto horse blood agar and into thioglycollate medium (48 hours, 37 °C).

Preparation of suspensions

50 per cent suspensions in distilled water (w/v) were made with a Potter Eveljhem apparatus. Acid fast bacilli (AFB) were counted by the method of Shepard (1960), using Wellcome plastic coated slides used for immunofluorescence. Aliquots of each suspension were inoculated onto horse blood agar and into thioglycollate broth.

Pretreatment of suspensions

Suspensions were pretreated with different concentrations of hydrochloric acid or sodium hydroxide followed or not by neutralization as indicated in table 2. If the procedure entailed neutralization, this was done after shaking for 10 min.

TABLE 2

Twelve different pretreatments (designated by letters b to u) of armadillo liver suspensions

Final concentrations (%)	HCl neutralized		NaOH neutralized	
	−	+	−	+
0.1	b		c	
0.2	t	s	u	
0.4		r		
0.8		g		h
1		q		
1.3		p		i
2		f		

a = no pretreatment.

Media

Löwenstein Jensen (LJ) and Ogawa (OG) media were used. The composition of the latter was described before (Pattyn and Portaels, 1980).

To Ogawa medium (table 3) were added before sterilization in the autoclave : either glucose, iron ammonioncitrate, tyrosine, veal brain extract prepared as described by Folch, Lees and Sloane Stanley (1957) or pyruvate. Suspensions of either *in vitro* grown mycobacteria, corynebacteria (Delville *et al.*, 1982), or *M. leprae* prepared from armadillo livers or human biopsies (final concentration 2 per cent) were also added. KH_2PO_4 at 3 per cent or 1 per cent were incorporated to Ogawa basal medium to give pH 5.5 and 6.0 respectively. Malachite green was omitted from some preparations.

TABLE 3

Ogawa medium modifications

N°	Additions	Na pyruvate	Bacterial suspensions	pH	M. G. (1)
OG1	Glucose (0.5 %)	—			+
OG2	$FeNH_4$ citr (0.1 %)	—			+
OG3	± tyrosine (0.5 %); ± glucose (2 %)	1 %	*M. phlei*	5.5, 6.0	+
OG4	Idem OG3	1 %	*M. lepraemurium* (liver)	5.5, 6.0	+
OG5	—	—	*M. lm* (liver, culture)	6	+
OG6	—	± 0.2 %	*M. leprae* (arm. liver, natural infection)	6	−
OG7	—	—	*M. leprae* (arm. liver, experimental infection)	5.5, 6.0	−
OG8	—	± 0.2 %	*M. leprae* cfr OG7	5.5, 6.0	−
OG9	—	± 0.2 %	*M. leprae* cfr OG7 or *M. leprae* (biopsies) or *M. lepraemurium* (liver)	6	−
OG10	—	± 0.2 %	*M. lufu* (2 strains) (2) or Corynebacterium sp. (3 strains)	5.5, 6.0	−
OG11	± calf brain (extract, lyophilized)	—	*M. phlei*	6.1	−

(1) MG : malachite green.
(2) Portaels (1980).

Inoculation of media

After inoculation with 0.4 ml liver suspension, tubes were incubated horizontally for 24 hours; they were then further incubated in vertical position at 30, 33 or 37 °C, some of them in humidified incubators in an atmosphere of 5 per cent CO_2; in the latter case stoppers were pierced with a 19 G needle (Portaels and Pattyn, 1981). Tubes were examined every fourthnight during one year.

Mouse foot pad (MFP) inoculation

Each liver suspension was titrated in MFP as described by Shepard (1960), with 5.10^3, 5.10^2, 50 and 5 AFB. A mouse was examined at six months and thereafter every three months, up to one year if the counts did not reach 5.10^5/MFP. When this figure was attained, five mice were examined.

Identification of in vitro *grown mycobacteria* was performed as described by Jenkins, Pattyn and Portaels (1982). The pH growth range was done and interpreted as described previously (Portaels and Pattyn, 1982).

Results

1. *Results of MFP inoculations* for livers 1, 2 and 3 are presented in table 4, and include the percentage of viable *M. leprae.* Results for liver 4 are not yet available.

TABLE 4

Results of titrations in mouse foot pads of AFB extracted from armadillo livers

	Number of AFB in inoculum				% viable AFB
	5.10^3	5.10^2	50	5	
Liver 1	4/5	0/5	0/5	0/5	0.026 %
Liver 2	0/5	0/5	0/5	0/5	< 0.02 %
Liver 3	5/5	2/5	1/5	0/5	2.4 %

2. *Non acid fast (NAF) bacteria*

Surface cultures of livers and the inside of their containers were negative for specimens 1 and 3 and revealed gross contamination for specimens 2 and 4. Cultures of the liver suspensions on blood agar and thioglycollate revealed that liver 1 contained non identified *Bacillus spec.*, liver 2 a mixture of *Salmonella* serovar 40 : $Z_4Z_{32}-$, *Staphylococcus saprophyticus* and *Micrococcus varians*, liver 3, *Micrococcus nishinomiyaensis* and liver 4 micrococci and *Corynebacterium* spec.

3. *Effect of pretreatments on the growth of NAF organisms*

Table 5 shows that only the more drastic pretreatment procedures (f, g, i, p and q) were able to reduce the percentage of contaminated tubes. Pretreatment by HCl followed by NaOH seems to be more effective : for sample 4 pretreatment h is followed by 68.2 per cent contaminations, pretreatment g reducing this figure to 10.1 per cent ($p < 0.01$; $\chi^2 = 91.78$). However for sample 2 there is no significant difference for pretreatments i and p.

TABLE 5

Number of tubes contaminated by non acid-fast bacteria after different pretreatments (NAFB)

Pretreatments (1)	Tubes contaminated/total tubes inoculated (%) Livers			
	1	2	3	4
a	39/40 (97.5 %)		46/200 (23.5 %)	5/6 (83.3 %)
b		73/74 (98.6 %)	5/32 (15.6 %)	
c				77/78 (98.7 %)
f	5/59 (8.5 %)	17/192 (8.9 %)		
g	6/40 (15.0 %)	8/139 (5.8 %)	8/52 (15.4 %)	18/178 (10.1 %)
h				58/85 (68.2 %)
i		6/111 (5.4 %)		13/72 (18.1 %)
p		6/71 (8.5 %)		
q			38/241 (15.8 %)	
r			8/136 (5.9 %)	
s				63/79 (79.7 %)
t	28/40 (70.0 %)			
u	38/52 (73.0 %)			

(1) See table 2.

4. *Primary* in vitro *cultures of AFB*

Livers 1 and 2 did not give rise to any visible growth of AFB, during one year of incubation. The samples from liver 3 (A5 and A10, table 6) gave rise to mycobacterial growth further designated as armadillo derived

TABLE 6

Cultivable *in vitro* mycobacteria isolated from armadillo livers

Pretreatments (2)	Tubes with cultivable mycobacteria/total uncontaminated tubes (%) Samples n°, liver 3 (1)		Samples n°, liver 4 (1)	
	A5	A10	A7	A8
a	5/40 (12.5 %)	3/114 (2.7 %)	0/1 (0 %)	
b		4/27 (14.8 %)		
c			0/1 (0 %)	
g	12/44 (27.3 %)		12/74 (16.2 %)	15/86 (17.4 %)
h			3/27 (11.1 %)	
i				6/58 (10.3 %)
r		3/128 (2.6 %)		
s			1/16 (6.3 %)	

(1) See table 1.
(2) See table 2.

mycobacteria (ADM). For both the A5 and A10 suspensions, pretreatment significantly enhanced growth of ADM. For A5 pretreatment g gave rise to 27.3 per cent positives versus 12.5 per cent without pretreatment ($p = 0.05$; $\chi^2 = 3.82$); for A10 pretreatment b, gave rise to 14.8 per cent positives versus 2.7 per cent without pretreatment ($p < 0.05$; $\chi^2 = 4.52$). Pretreatment r, involving neutralization however is inferior to pretreatment b. It thus seems that pretreatment with HCl without ensuing neutralization is more growth promoting than with neutralization. Results obtained with suspensions A5 and A10 cannot be compared since they were inoculated onto different media. Suspensions A7 and A8 prepared from liver n° 4 also gave rise to mycobacterial growth. Differences between pretreatment g and other were not significant in this case.

No significant differences were observed between the incubation temperatures.

Humidified CO_2 enriched atmosphere was not beneficial.

On LJ medium only one colony was obtained from suspension A10, after pretreatment b, and after two months incubation at 33 °C (table 7). Since pretreatment b involved the addition of HCl to a final concentration of 0.1 per cent without neutralization, the inoculum may have lowered the pH of the medium.

Ogawa medium at pH 6 and 5.5 gave rise to growth of AFB in all cases (table 7) except modification OG 4. The number of colonies was influenced by the composition of the medium : on regular Ogawa the number of colonies varied between 1 and 10, with pyruvate added there was no growth or only a single colony. Media producing more than 10 colonies were without pyruvate but contained autoclaved suspensions of bacteria (*Mycobacterium leprae*, *Mycobacterium lepraemurium*, *Mycobacterium lufu*, *Mycobacterium phlei* or corynebacteria). The ADM developed at both pH 5.5 and 6.0, and in the presence or absence of malachite green. Incubation times varied between 1 and 3 months. The shortest incubation time was obtained for suspension A5 after acid pretreatment followed by neutralization on medium OG 5 (Ogawa with *M. lm.* added).

5. *Characterization of ADM*

Subcultures were made on LJ and Ogawa in logarithmic dilutions starting with a suspension containing 10^9 AFB/ml. Inocula below 10^6 AFB/ml did not grow on LJ. On Ogawa medium, 10^5 AFB corresponded to 1 colony forming unit. Growth was better at 37 °C than at 33 °C.

For the identification tests, suspensions of 10^9 AFB/ml were used. From suspensions A5 and A10 from liver n° 3, and A7 and A8 from liver n° 4, three slightly different types of mycobacterial strains could be distinguished. Their characters are presented in table 8, together with those of *Mycobacterium avium*, *Mycobacterium scrofulaceum* and *Mycobacterium gordonae*. Results of catalase and tween hydrolysis tests bring some of these ADM close to *M. scrofulaceum* (8480 and 8489) or *M. gordonae* (8251 and 8263). They differ from these however by not growing on LJ, and their sensitivity to INH and hydroxylamine. Strains 8233 and 8335 are intermediary strains belonging to the *M. avium-intracellulare-scrofulaceum*

TABLE 7

Mycobacterial growth on Ogawa (OG) and Loewenstein Jensen (LJ) media

Samples and pretreatments			AFB/tube	LJ	OG	Ogawa modifications (1)								
						3	4	5	6	7	8	9	10	11
	A5	a	$2.5 \cdot 10^7$	c	c	1	–	–	–	+	1	1		
	A5	g	10^7	–	1	1	–	#	+	#	1			
3	A10	a	$4.6 \cdot 10^6$	–	–	–	–			c	1	–		–
	A10	b	$8 \cdot 10^6$	1	–	c	–				–	–		1
	A10	r	$3 \cdot 10^6$	–	–	–	–				–	–		–
	A7	g	$5 \cdot 10^7$	–	1							–	#	
	A7	h	$5 \cdot 10^7$	c	+						c	c	#	
4	A7	s	$5 \cdot 10^7$	–	c						c	c	1	
	A8	g	$8 \cdot 10^7$	–	–								#	#
	A8	i	10^8	–	+								#	#

(1) See table 3.

Blanc spaces = not tested; c = contaminated; 1 = one colony/tube; + = 2 to 10 colonies/tube; # = > 10 colonies/tube.

TABLE 8

In vitro **characteristics of cultivable mycobacteria isolated from armadillo livers**

Strains n°		LJ pigment (1)		Growth at (°C) 37	42	Resistance to (3) I	T	H	P	Catalase 45 mm ∧	Nitratase red.	Acid phosphatase	Tween hydrolysis	Urease	Putrescine	Colony on OAA (4)
	8233	–	Sc	+	–	–	+	–	+	–	–	–	–	+	–	Sm S
Liver 3	8480	–	Sc	+	–	–	+	–	+	+	–	–	–	+	–	Sm S
	8251	–	Sc	+	–	–	+	–	+	+	–	–	+	+	–	Sm S
	8335	–	Sc	+	–	–	+	–	+	–	–	–	–	+	–	Sm S
Liver 4	8489	–	Sc	+	–	–	+	–	+	+	–	–	–	+	–	Sm S
	8263	–	Sc	+	–	–	+	–	+	+	–	–	+	+	–	Sm S
M. avium (2)		+	–	+	+	+	+	+	+	–	–	–	–	–	–	SmS-SmT
M. scrofulaceum (2)		+	Sc	+	+	+	+	+	+	+	–	–	–	+	–	SmS-SmK
M. gordonae (2)		+	Sc	+	–	–	+	+	+	+	–	+	+	+	–	SmS-SmK

Sc : scotochromogenic.

(1) No growth with $< 10^6$ AFB.

(2) Data from Jenkins *et al.*

(3) 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.

(4) OAA = oleic acid albumin agar. For colony morphology descriptions, see Jenkins *et al.* (1982).

(MAIS) complex (Portaels, 1978). Figure 1 shows the pH range of growth for the strains isolated from liver 3, in comparison with *M. avium*, *M. scrofulaceum* and *M. gordonae* (Portaels & Pattyn, 1982). Strain 8480 has an identical pH growth range as *M. scrofulaceum* with the optimum between 5.4 and 6.5. The optimal pH growth ranges for strains 8233 and 8251 are identical and extremely narrow (5.4-5.7), and very distinct from the optimal pH of strains belonging to the MAIS complex (Portaels, unpublished results) and *M. gordonae.* The optimal pH growth range of strain 8335 from liver 4 is identical to that of strains 8233 and 8251 (5.4-5.7).

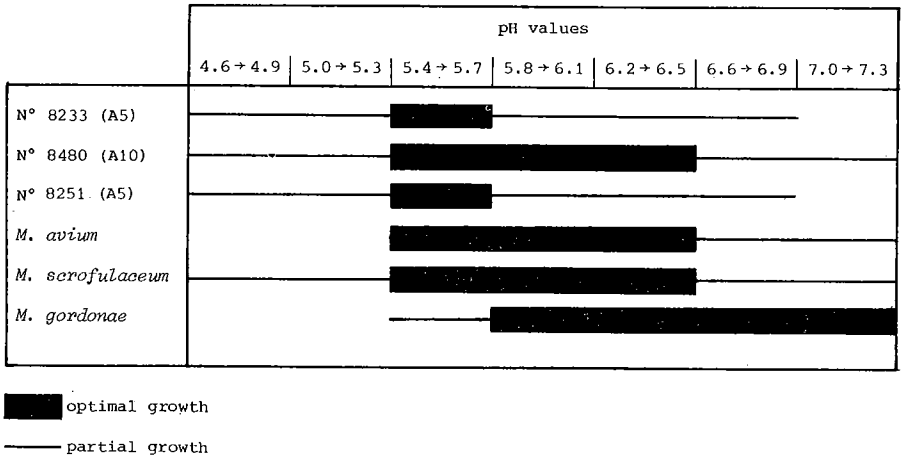

Figure 1.
pH growth range of cultivable mycobacteria isolated from armadillo livers.

Discussion

We showed that armadillo livers may contain a variety of non AFB. Surface sampling is insufficient for sterility testing since such cultures may remain negative for 48 hours while the suspensions give rise to numerous colonies (table 5) even after some pretreatments. Sterility testing should be done for a much longer time, at least two weeks.

There are several publications on the isolation of *in vitro* growing mycobacteria from infected armadillo tissues. Nakamura, Itoh & Waki (1976) isolated a scotochromogen on Ogawa medium from an armadillo leproma. Kirschheimer (1979) isolated a mycobacterium from a lymphnode of an uninoculated animal. Nishiura *et al.* (1980) isolated *M. scrofulaceum* and *M. intracellulare* from the spleen of an animal with natural leprosy. Prabakharan, Harris & Kirschheimer (1980) isolated cultivable AFB from an uninoculated animal.

The present paper is the first to show the presence of high numbers of scotochromogenic AFB in *M. leprae* containing armadillo livers, and raises a number of questions.

1. Were these mycobacteria introduced in the armadillos after or before they were killed ? Were they present before the inoculation of the animals with *M. leprae* ?
2. Are their numbers sufficiently important to influence the results of studies performed on purified *M. leprae* suspensions prepared from such tissues ?
3. To what species do these mycobacteria belong ? Is there any relationship with *M. leprae* ?

Question 1

The first hypothesis was that these strains were laboratory contaminants. However, samples from the same organ, manipulated at widely different moments gave rise to the same mycobacteria. Five months separated the work with samples A5 and A10 from liver n° 3, and two months elapsed between manipulation of samples A7 and A8 from liver n° 4.

During the same period a great number of human biopsy specimens and MFP harvests (98 and 127 respectively) were handled along similar though slightly different techniques and in no instance a colony was observed among about 2,000 culture tubes. We therefore think that the cultivable AFB were present in the liver tissue.

The growth at acid pH suggests that these strains can survive within the phagolysosomes of macrophages, as is the case for *M. lepraemurium* (Hart *et al.*, 1972; Lowrie, Aber & Jacket, 1979; Trouet, Tulkens & Schneider, 1980), *M. leprae* (Hart *et al.*, 1972; Imaeda, 1965) and *Leishmania* spec. (Jones & Masur, 1980; Chang, 1980; Lewis & Peters, 1977). According to Chang (1980) the intralysosomal pH of *Leishmania* infected macrophages is 5.4-5.7, precisely the optimal pH range for the strains isolated in the present study.

As is the case for *M. lepraemurium*, another intracellularly multiplying « difficult to grow mycobacterial species », primary in vitro culture are favoured by pretreating organ suspensions with acid or alcali (Portaels, 1981). It is likely that in both cases such pretreatments alter some factor(s) surrounding the organisms in vivo which limit their in vitro multiplication.

The narrow acid pH growth range and the beneficial effect of the pretreatments are in favour of the argument that these bacteria are present, intracellularly, in the armadillo livers.

If the ADM are present in the armadillo livers, the question of their origin arises. They may have been injected in the animals at the time of the injection of human derived *M. leprae* or they present before or were introduced later on.

Numerous authors have isolated different kinds of mycobacteria from human leprous tissues, particularly scrotochromogens. However the scotochromogenic ADM we isolated have special characters particularly with respect to the pH growth range. We are not aware of any systematic effort to isolate mycobacteria from human leprous tissue applying the methods of the present study. For these reasons the presence of contaminating « ADM » in the human inocula can not be excluded at present.

ADM may also have been present in the armadillos before the experimental infection. They may be present in wild animals or introduced in

armadillos kept in captivity via non sterile drinking water, litter or food. For these reasons, organs of wild caught armadillos as well as organs of uninoculated armadillos kept in capacity should be studied for the presence of mycobacteria similar to those we cultivated.

Question 2

As more than 10 colonies of ADM developed on some media (table 7), with 10^5 AFB corresponding to 1 CFU on Ogawa medium, inocula did contain 10^6 and more ADM. These results implicate that more than 10 per cent of the AFB in livers 3 and 4 were ADM. Thus, they constituted a non negligible fraction and their presence together with *M. leprae* may influence the results of studies on *M. leprae* purified from such livers. Detailed studies are presently under way to quantify more precisely the numbers of ADM in livers 3 and 4.

Question 3

There was a correlation between the viability of *M. leprae* in the armadillo livers as measured by MFP inoculation and the in vitro growth of ADM. Livers n° 1 and n° 2 containing very small fractions of living *M. leprae*, as shown by MFP inoculations, did not give rice to in vitro growth of ADM, while liver n° 3, containing a much higher proportion of viable *M. leprae* produced ADM. Unfortunately results of liver n° 4 in MFP are not yet available.

All the strains differ from all presently known mycobacteria. With their very narrow optimal acid pH growth range they may well belong to a new species. However more detailed studies are necessary to define their relationship with other mycobacteria and also amongst themselves.

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Bacteriologische studies van armadillo levers besmet met *Mycobacterium leprae*.

Samenvatting — Niet-mycobacteriën en *in vitro* kweekbare mycobacteriën werden in groot antal geïsoleerd uit twee op vier armadillo levers besmet met menselijke *Mycobacterium leprae*. Deze kiemen waren « moeilijk kweekbare mycobacteriën » en *in vitro* vermenigvuldiging was slechts mogelijk wanneer de volgende drie voorwaarden vervuld werden : (1) de inocula moeten meer dan 10^5 levende kiemen bevatten; (2) de suspensies moeten voorbehandeld worden met NaOH of HCl; (3) zure media met pH 5.4-5.7 die geautoclaveerde mycobacteriën suspensies bevatten moeten gebruikt worden.

De aanwezigheid van deze mycobacteriën in armadillo levers zou de resultaten kunnen beïnvloeden van studies met *M. leprae* gezuiverd uit deze organen. De verhouding van mycobacteriën met *M. leprae* moet verder worden bestudeerd.

Etudes bactériologiques sur des foies de tatous infectés par *Mycobacterium leprae*.

Résumé — Des études bactériologiques réalisées sur 4 foies de tatous infectés par *M. leprae*, ont donné lieu dans 2 cas à la culture d'un grand nombre de mycobactéries et de contaminants non mycobactériens.

Les mycobactéries isolées à partir de ces foies de tatous sont des organismes de culture difficile. Trois conditions doivent être respectées : (1) l'inoculum doit contenir un nombre élevé de bactéries ($> 10^5$); (2) les suspensions de foie doivent être soumises à des traitements préalables au NaOH ou à l'HCl; (3) les milieux de culture doivent être acides (pH entre 5.4 et 5.7) et enrichis de suspensions autoclavées de mycobactéries.

La présence de ces mycobactéries dans les foies de tatous infectés par *M. leprae*, peut influencer les résultats des recherches réalisées sur *M. leprae* purifié à partir de tels organes.

Des études taxonomiques plus approfondies sont nécessaires afin de déterminer la position systématique de ces mycobactéries dérivées de tatous, par rapport aux autres mycobactéries et en particulier *M. leprae*.

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REFERENCES

Chang, K. P. (1980) : Endocystosis of Leishmania-infected macrophages. Fluorometry of pinocytic rate, lysosome-phagosome fusion and intralysosomal pH. *in* The Host Invader Interplay, ed. H. Van den Bossche. Elsevier/North-Holland Biomedical Press, Amsterdam, 231-234.

Delville, J., de Sloovère, T., Fontaine, F., Gueur, M. C., Rajjan, W., Spina, A., Cocito, C. (1982) : Biological properties of diphteroïd bacteria (LDC) isolated from human leprosy lesions. Ann. Microbiol. (Inst. Pasteur), **133B**, 159-160.

Folch, J., Lees, M., Sloane Stanley, G. H. (1957) : A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem., **226**, 497-509.

Hart, P. d'Arcy, Armstrong, J. A., Brown, C. A., Draper, P. (1972) : Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infect. Immun., **5**, 803-807.

Imaeda, T. (1965) : Electron microscopy. Approach to leprosy research. Int. J. Lepr., **33**, 669-683.

Jenkins, P. A., Pattyn, S. R., Portaels, F. (1982) : Diagnostic bacteriology. *in* The Biology of the Mycobacteria. eds. C. Ratledge and J. L. Stanford. Academic Press, London, 441-471.

Jones, T. C., Masur, H. (1980) : Survival of *Toxoplasma gondii* and other microbes in cytoplasmic vacuoles. *in* The Host Invader Interplay. ed. H. Van den Bossche. Elsevier/North-Holland Biomedical Press, Amsterdam, 157-164.

Kirschheimer, W. F. (1979) : Examination of North American armadillos for mycobacteriosis. A further report. Lepr. India, **51**, 60-64.

Lewis, D. H., Peters, W. (1977) : The resistance of intracellular Leishmania parasites to digestion by lysosomal enzymes. Ann. Trop Med. Parasit., **71**, 295-312.

Lowrie, D. B., Aber, V. R., Jacket, P. S. (1979) : Phagosome-lysosome fusion and cyclic adenosine 3' : 5'-minophosphate in macrophages infected with *Mycobacterium microti*, *Mycobacterium bovis* BCG of *Mycobacterium lepraemuium*. J. Gen. Microbiol., **110**, 431-441.

Nakamura, M., Itoh, T., Waki, C. (1976) : Isolation of a cultivable mycobacterium from an armadillo subcutaneous tissue infected with *M. leprae* and characterization of this isolated strain. La Lepro, **45**, 217-222.

Nishiura, M., Fukunishi, Y., Okada, S., Abe, M., Kohsaka, K., Yoneda, K., Mori, T., Ito, T., Bretana, A., Convit., J., Walsh G. P., Meyers, W. M., Binford, C. H. (1980) : A joint study on the naturally acquired leprosy-like disease of armadillos. Int. J. Lepr., **48**, 493-494.

Pattyn, S. R., Portaels, F. (1980) : In vitro cultivation and characterization of *Mycobacterium lepraemurium*. Int. J. Lepr., **48**, 7-14.

Pattyn, S. R., Portaels, F. (1981) : Growth of *M. leprae* in experimental animals and on artificial media. Bull. Inst. Pasteur, **79**, 233-250.

Portaels, F. (1978) : Difficulties encountered in identification of *M. avium*, *M. intracellulare*, *M. scrofulaceum* and related strains. Amer. Rev. Resp. Dis., **118**, 969.

Portaels, F. (1980) : Unclassified mycobacterial strains susceptible to dapsone isolated from the environment in Central Africa. Int. J. Lepr., **48**, 330.

Portaels, F. (1981) : In vitro cultivation of *M. lepraemurium* and *M. leprae*. Lepr. India, **53**, 675-676.

Portaels, F., Pattyn, S. R. (1981) : Factors influencing the in vitro growth of *M. lepraemurium*. Int. J. Lepr., **49**, 194-197.

Portaels, F., Pattyn, S. R. (1982) : Growth of mycobacteria in relation to the pH of the medium. Ann. Microbiol., **133B**, 213-221.

Prabhakaran, K., Harris, E. B., Kirschheimer, W. F. (1980) : Failure to detect o-diphenoloxidase in cultivable mycobacteria obtained from feral armadillos. Lepr. Rev., **51**, 341-349.

Shepard, C. C. (1960) : The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. J. Exp. Med., **112**, 445-454.

Trouet, A., Tulkens, P., Schneider, Y.-J. (1980) : Subcellular localization of infectious agents : pharmacological and pharmacokinetic implications. *in* The Host Invader Interplay. ed. H. Van den Bossche. Elsevier/North Holland Biomedical Press, Amsterdam, 31-44.

Walsh, G. P., Storrs, E. E., Meyers, W., Binford, C. H. (1977) : Naturally acquired leprosy-like disease in the nine banded armadillo *(Dasypus novemcinctus)* : recent epizootiologic findings. J. Reticuloendothel. Soc., **22**, 363-367.