PN-ABQ-412
10.099

FINAL REPORT

September 24, 1990 - October 31, 1993

Submitted to the Office of the Science Advisor
U.S. Agency for International Development

Rapid *In Vitro* Detection of Drug-Resistant *Mycobacterium leprae*

Principal Investigator: Dr. Gerald P. Walsh

Co-Principal Investigators: Dr. Eduardo Dela Cruz
Dr. T. T. Fajardo
Dr. Roland V. Cellona
Dr. Rodolfo M. Abalos

Grantee Institution:
Leonard Wood Memorial (American Leprosy Foundation)
Rockville, Maryland/Cebu City Philippines

Collaborator: Dr. Robert Hastings

Gillis W. Long Hansen's Disease Center
Louisiana State University
Baton Rouge, Louisiana

Project Number: 10.099
Grant Number: DHR-5600-G-00010100

A.I.D. Grant Project Officer: Dr. Howard A. Minners

Project Duration: September 24, 1990 - October 31, 1993

DEC 27

cc: BOSTID, CDIE

II. TABLE OF CONTENTS

		Page
I.	COVER SHEET	1
II.	TABLE OF CONTENTS	2
III.	EXECUTIVE SUMMARY	3
IV.	RESEARCH OBJECTIVES	4-5
V.	METHODS AND RESULTS (with Tables)	5-9
VI.	IMPACT, RELEVANCE & TECHNOLOGY TRANSFER	10
VII.	PROJECT ACTIVITIES/OUTPUTS	10-11
VIII.	PROJECT PRODUCTIVITY	11
IX.	FUTURE WORK	11
X.	LITERATURE CITED	11

III. EXECUTIVE SUMMARY

The goal of this project was to develop and establish an in vitro method for detecting viability of Mycobacterium leprae in clinical isolates and comparing this technology with the conventional mouse footpad system, which is technically demanding and time-consuming.

The first phase of the project focused on procurement of equipment and training of technical personnel. Acquiring the necessary equipment involved purchase and shipment of major items from the United States to Cebu, Philippines. Training of technical personnel at the Cebu Center initially involved demonstration by our U.S. collaborators and subsequently, performance of the in vitro assay by technical personnel at the Center. The later effort in this phase of the project also involved optimization studies of the in vitro assay using human-derived biopsy specimens since the original technology was developed using nude mouse-derived M. leprae.

The second phase of the project focused on comparison of the in vitro assay with the conventional mouse footpad system in clinical studies to determine the effect of new drugs on the viability of M. leprae and ultimately, on the course of the disease. To achieve this, we initiated a study evaluating the effect of Minocycline in patients. Early laboratory studies suggested Minocycline was effective in inhibiting multiplication of M. leprae. The results of our study clearly demonstrated a good correlation between results of the in vitro assay and the mouse system for detecting loss of viability of M. leprae.

A paper describing these results was presented at the XIV International Leprosy Congress (Orlando, FL, August 1993) and a manuscript has been submitted for publication.

The in vitro assay technology has been effectively transferred to Cebu and, when appropriate, will be incorporated into future studies.

IV. RESEARCH OBJECTIVES

In spite of efforts spanning many decades, Mycobacterium leprae has not yet been successfully cultured in the laboratory. The discovery in the 1960's of limited multiplication of M. leprae in the mouse footpad provided a system for detecting viability of leprosy bacilli, as well as, for determining drug sensitivity of isolates from patients with active, untreated leprosy (1). Although the mouse model was a major breakthrough, the procedure is demanding and time consuming.

This project was undertaken to develop and establish an in vitro method for detecting viability by measuring the metabolic activity of M. leprae using clinical isolates obtained from untreated multibacillary leprosy patients. The second phase of our studies was to compare this assay with the mouse footpad system, the conventional method used for detecting viability and drug sensitivity of M. leprae. In addition to being technically demanding and time consuming, the mouse model is expensive and it was our goal to determine if an in vitro assay would provide the same information in a shorter period of time, and using technology less demanding than that required for the mouse footpad system. Earlier studies using laboratory isolates of M. leprae from nude mice had demonstrated the feasibility of the in vitro assay (2) and our objectives were to further develop the assay and establish the technology in a research facility located in a leprosy-endemic area of a developing country.

The development of the in vitro assay was unique and innovative because it relied on the utilization of radiorespirometry measuring metabolic activity of M. leprae rather than the in vivo bacillary multiplication necessary in the mouse footpad model.

The Leonard Wood Memorial has been designated as a World Health Organization (WHO) Collaborating Center in Research and Training in Leprosy and is, therefore, frequently involved in WHO

sponsored clinical evaluations of new drugs and drug regimens proposed for use in leprosy. In the early training and optimization of the in vitro assay, patients recruited and admitted for WHO-sponsored studies served as a source of clinical isolates of M. leprae needed for this project.

From a financial standpoint, the Leonard Wood Memorial provided the funds needed to cover expenses that exceeded the USAID funds approved and budgeted for this project.

V. METHODS AND RESULTS

Earlier laboratory studies reported that Minocycline was effective in inhibiting multiplication of M. leprae in the mouse footpad (3). Fourteen patients were recruited for the Minocycline study which involved three regimens 1) 100 mg daily for 6 months 2) 200 mg daily for 30 days followed by 100 mg daily for 5 months and 3) intermittent dosaging with 100 mg for 6 days during the first month, followed by 100 mg daily for 5 months. Table I (p.7) provides characteristics and regimens of the patients recruited for this study.

Clinically, improvement was noted as early as 3 days particularly in those patients presenting with papulo-nodular and plaque-type lesions. Improvement was visibly less dramatic after 30 days. Of particular importance was that no reactional episode occurred during the 6 months of treatment suggesting an anti-inflammatory effect of Minocycline. The clinical response of the patients on the various regimens is detailed in Table II (p.8) which also includes changes in the Bacterial Index (BI) after 6 months of therapy.

Biopsy specimens for mouse footpad inoculation and for the radiorespirometric in vitro assay were taken from each patient at days 0,3,7,10,14,21,30, and finally at day 180, the end of treatment. To minimize variation, each biopsy specimen was divided and one half was used for mouse inoculation, and the remaining half

processed for the in vitro assay. Harvests of inoculated mice were performed 8-12 months after inoculation.

Specimens for the in vitro assay were processed using the standard methods described earlier (2). Briefly, bacilli from a ground suspension were centrifuged, resuspended in 7H12 media and counted. To measure the oxidation of palmitic acid to CO_2, aliquots were added to 7H12 media containing ampicillin and amphotericin B (inhibitors of non-M. leprae organisms). To this mixture, ^{14}C radiolabelled palmitic acid was added. After 1 week incubation, the inner vial of bacilli was removed and evolved $^{14}CO_2$ was measured by adding scintillation fluid and determining CPM's in a Beckman LS-5801. Data are presented as the mean CPM of replicate samples corrected for background activity by subtraction of the mean CPM from heat-killed samples.

In all patients, radiorespirometric values declined with treatment. Although the correlation between the in vitro assay and the mouse footpad data was not always absolute, in most cases a sharp decline in mouse footpad viability between two fine points was also reflected in the results of the in vitro assay. An obvious advantage of the radiorespirometry is the generation of quantitative data within one week of biopsy in contrast to 1 year incubation in order to detect growth in the mouse footpad. Table III (p.9) details the clinical and bacteriologic effects and includes the comparison of mouse footpad and the in vitro assay. Also included in this table are the results of phenolic glycolipid I (PGL-I) antigen studies. PGL-I antigen positivity is lost in the presence of effective therapy in leprosy, so this parameter was also included in the study.

As indicated in the Table of Contents, Tables I, II and III follow as Pages 7, 8 and 9.

Table I. Pre-therapy characteristics of patient population, and minocycline dose.

Name	Age Sex	Dis Dur	Clin Dx	Histo Dx	Ave. BI	Minocycline Dose
New Untreated:						
LN	15/F	8 yr	LL	LLp	4.8	Intermittent*
QE	23/M	1.5 yr	LL	LLp	4.3	100mg./day x 6 mos.
CL	13/F	1 yr	LL	LLs	5.0	Intermittent*
IC@	19/F	3 yr	LL	LLs	5.0	200mg./day x 1 mo. 100mg./day x 5 mos.
EL	27/F	1 yr	LL	LLs	5.5	100mg./day x 6 mos.
BJ	27/M	3 yr	LL	LLp	4.8	100mg./day x 6 mos.
TP	15/M	1 yr	LL	LLs	5.0	100mg./day x 6 mos.
CF	14/M	3 yr	LL	LLs	5.0	100mg./day x 6 mos.
FS	19/M	5 yr	LL	LLs	5.0	200mg./day x 1 mo. 100mg./day x 5 mos.
FM	21/F	1 yr	BL	BL	4.5	200mg./day x 1 mo. 100mg./day x 5 mos.
Relapsed:						
JM**	59/M	50 yr	LL	LLs	3.5	100mg./day x 6 mos.
SU	31/M	5 yr	LL	LLs	5.0	100mg./day x 6 mos.
PB	31/M	23 yr	LL	Hist.Nod	3.3	100mg./day x 6 mos.
RE	38/F	28 yr	LL	LLs	4.7	200mg./day x 1 mo. 100mg./day x 5 mos.

* - 100 mg. daily, 3 days, (1,2,3) no treatment Day 4-14, 100 mg. daily, 3 days, (15,16,17) no treatment Day 18-30 (CL), no treatment Day 18-42 (LN), then 100 mg. daily for 5 months

** - absconded, treatment only for 72 days

@ - no pre-therapy mouse footpad inoculation

Table II. Clinical and bacteriologic (Ave. BI and LIB) results for patients completing 6 months of therapy.

Dose/Duration	No. Pts.	Clinical Status■		Bacteriology			
				Ave. BI		LIB	
		Isl	Imod	Prel.	Final	Prel.	Final
200 mg/day 1Mo. 100 mg/day 5Mo.	3**	1	2	4.73	4.33	4.13	4.35
100 mg/day 6Mo.	7*	4	3	4.70	4.60	5.75	5.18
Intermit 1Mo. 100 mg/day 5Mo.	2	1	1	4.90	4.65	5.90	5.72
Total	12	6	6	4.78	4.54	5.37	5.06

* - one patient (JM) absconded and is not included in the above tabulation.

** - one patient (IC), no pre-therapy MFP inoculation result, not included in the above tabulation.

■ - Isl - slight improvement, Imod - moderate improvement

Table III

CLINICAL AND BACTERIOLOGIC EFFECTS OF VARIOUS MINOCYCLINE DOSAGES

	Day 0			Day 3			Day 7			Day 10			Day 14			Day 30			Day 180			
Pt.	MFP +/total	CPM**** (SD)	PGL-1	MFP +/total	CPM (SD)	PGL-1	MFP +/total	CPM (SD)	PGL-1	MFP +/total	CPM (SD)	PGL-1	MFP +/total	CPM (SD)	PGL-1	MFP +/total	CPM (SD)	PGL-1	MFP +/total	CPM (SD)	PGL-1	Clin*** Eval
200 MG/DAY x 1 MONTH; 100 MG/DAY x 5 MONTHS																						
FS	7/7	4533 (929)	2+	9/9	4424 (1147)	3+	9/9	5545 (1090)	2+	9/9	4314 (1249)	2+	9/9	1928 (427)	2+	0/9	379 (71)	1+	0/10	--	Neg	Isl
FM	9/9	3019 (336)	3+	9/10	3182 (932)	3+	9/9	2883 (607)	2+	9/10	3029 (454)	2+	4/7	1535 (352)	2+	2/9	934 (150)	2+	0/10	21 (27)	Neg	Imod
RE	8/9	3257 (1242)	2+	10/10	--	--	9/10	5718 (2396)	1+	7/10	--	1+	5/10	1970 (747)	2+	2/10	400 (230)	1+	0/9	-47 (39)	Neg	Imod
100 MG/DAY x 6 MONTHS																						
EL	2/9	750 (230)	1+	--	--	--	1/10	67 (40)	--	6/7	77 (56)	--	7/10	925 (265)	--	0/8	44 (38)	1+	0/10	--	Neg	Imod
TP	7/8	5296 (1746)	2+	--	--	--	10/10	1869 (349)	2+	8/10	610 (171)	2+	3/10	289 (114)	1+	0/9	10 (26)	1+	0/10	--	Neg	Isl
CF	6/7	2024 (214)	2+	--	--	--	5/10	523 (241)	2+	5/10	230 (54)	2+	0/9	309 (76)	2+	1/10	0 (14)	Neg	0/10	307 (225)	Neg	Isl
BJ	9/9	521 (86)	--	--	--	--	7/9	492 (49)	--	1/9	388 (72)	--	0/9	314 (112)	--	0/9	-9 (11)	--	0/9	-16 (17)	--	Isl
QE	7/8	6787 (356)	--	8/10	9279 (476)	--	7/8	3446 (1318)	--	--	--	--	2/10	686 (205)	--	0/10**	22** (14)	--	0/8	-13 (11)	--	Imod
PB	11/11	4209 (1411)	--	--	--	--	--	--	--	--	--	--	0/10	55 (6)	--	0/9	0 (150)	--	0/10	--	--	Isl
SU	8/9	723 (56)	--	--	--	--	--	--	--	--	--	--	--	.	--	0/9	9 (10)	--	--	--	--	Imod
INTERMITTENT*																						
LN	8/9	5026 (1455)	--	9/10	769 (36)	--	9/9	2540 (777)	--	--	--	--	9/9	1594 (388)	--	6/10**	43** (3)	--	0/9	-3 (11)	--	Isl
CL	8/9	2279 (1191)	--	9/9	4463 (1832)	--	9/9	--	--	--	--	--	9/9	3492 (1160)	--	5/8	131 (63)	--	0/9	-9 (13)	--	Imod

**** Mean count per minute/10E6 AFB (standard deviation)
*** Isl - slight improvement; Imod - moderate improvement
** Day 42

* LN - 100 mg/day on days 1,2,3,15,16,17,43-180
CL - 100 mg/day on days 1,2,3,15,16,17,31-180
-- - Not Done

VI. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

Leprosy remains a significant public health problem in the Philippines as well as in many developing countries. There are an estimated 60,000 to 80,000 cases in the Philippines. Although Multidrug Therapy (MDT) has been effective in reducing prevalence, the incidence of the disease (i.e., new cases reported) has not decreased.

The development and establishment of the in vitro assay involved the transfer of technology to a Center in a leprosy endemic area of a developing country. The training required for such a transfer has added to the sophistication of the Center resulting in an increase in the expertise and capability of the LWM professional and technical staff involved in the project.

The results of the study provided basic confirmation that the in vitro assay is a valuable tool for assessing viability of M. leprae and will be incorporated into larger studies in which viability of M. leprae must be determined. We believe, however, additional studies that include the in vitro assay as well as the mouse model must be done before the mouse model can be discontinued.

The project required the purchase of 3 major pieces of equipment: a liquid scintillation counter, a biosafety cabinet and a CO_2 incubator. Because of the availability of this equipment the Immunology Unit at the Center has been able to initiate studies involving the use of Lymphoproliferative Assay (LPA) and related technology, requiring this equipment. Such studies would not have been possible without this grant-funded equipment.

VII. PROJECT ACTIVITIES

1. T.T. Fajardo, L. Villahermosa, E.C. dela Cruz, R. Abalos, S. Franzblau and G.P. Walsh. Minocycline in the treatment of lepromatous leprosy. XIV International Leprosy Congress, Orlando, FL. August, 1993 (Abstract).

2. T.T. Fajardo, L. Villahermosa, E.C. dela Cruz, R. Abalos, S. Franzblau and G.P. Walsh. Minocycline in lepromatous leprosy. (Manuscript submitted for publication)

VIII. PROJECT PRODUCTIVITY

The project accomplished all of the proposed goals which was to further develop the in vitro assay by transferring the technology to a Center in a leprosy endemic area and to assess the capacity of the assay to monitor the viability of M. leprae comparing it to the conventional mouse footpad technique.

IX. FUTURE WORK

The in vitro assay will be used in future studies in which detection of viability is a primary parameter. In spite of the good correlation between the in vitro assay and the mouse footpad system, we believe additional comparisons are needed before we will consider discontinuing the mouse model.

It is noteworthy that although the original goal of the grant states that the in vitro assay would be used to detect drug resistant M.leprae, we realized that more basic studies (optimization of the technology, etc.) and a one-to-one comparison with the mouse footpad was a greater priority than detecting drug resistant M.leprae. This change in emphasis was reported in our Year One Progress Report.

X. LITERATURE CITED

1. Shepard, C.C. Multiplication of M. leprae in the footpad of the mouse. Int. J. Lepr. 30:291-306, 1962.

2. Franzblau, S.G. Oxidation of palmitic acid by Mycobacterium leprae in an axenic medium. J. Clin. Micro. 26:18-21, 1988.

3. Gelber, R.H. Activity of minocycline in Mycobacterium leprae-infected mice. J. Infect. Dis. 156:236-239, 1987.