

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose *VO*
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C5-109

Attachment

U.S.-Israel CDR Project C5-109

Rapid Detection of Drug-Resistant and Viable *Mycobacterium leprae* in the Patient with Lepromatous Leprosy--Report of Progress 1 January - 30 September 1989

In summary, during the past nine months, work was concentrated, as during the preceding period, upon development of a simple method for partial purification of *M. leprae*, upon development of the LAMMA (laser assisted microprobe mass analysis) technique as a probe for viable *M. leprae*, and upon the initiation of collaborative work with Dr. Niwat Montreewasawat, Raj-Pracha-Samasai Institute, Bangkok, Thailand.

Simple purification method. During the past year, efforts have concentrated on treatment of the organisms by enzymes, the purpose being to purify the organisms sufficiently to permit radioautography and electron-microscopy, when the number of organisms is limited, without suffering intolerable losses, and without compromising the viability of the organisms.

Because the *M. leprae* to be subjected to autoradiography and electron-microscopy are harvested from, and, therefore, contaminated by host-tissue, some purification is required before the analyses may be carried out. Earlier work in the laboratory of Prof. U. Seydel, Forschungsinstitut Borstel, Borstel, FRG, had resulted in the development of a purification protocol, involving successive incubations with lipase and a mixture of proteases. Employing *M. lepraemurium* (MLM) as a surrogate for *M. leprae*, we have modified the protocol, to minimize the number of centrifugations and washes required, thus minimizing the loss of organisms, testing the modifications both by "titrating" the viable organisms in mice, and by LAMMA analysis (in Borstel) of the intracellular ratios of $[Na^+]$ to $[K^+]$ ions. As shown in Table 1, the first purification protocol, protocol "C", consisting of incubation with lipase for 90 min, followed by incubation with a mixture of collagenase, trypsin and chymotrypsin for 30 min, achieved excellent purification, but resulted in a considerable loss of viability, indicated by both the LAMMA measurement and inoculation of mice. The purification protocol was then modified by successive reductions of the periods of incubation with the enzymes, each modification being tested both by LAMMA-analysis and by mouse-

Table 1. Effect of purification on viability of MLM measured by LAMMA and by titration in mice

Experiment	Purification											
	A*		C		E		F		G		H	
	MPN ⁺	MPN	Na/K	MPN	Na/K	MPN	Na/K	MPN	Na/K	MPN	Na/K	
1	0.08	0.008	>1									
2	0.014	0.0064	>1									
3	0.20	---	---	0.059	1.2	0.0025	1.5	0.0039	0.6	0.0018	1.2	
4	≥0.48	---	---	0.17	0.38	0.068	0.45	≥0.48	0.51	≥0.48	0.41	

*A = control (unpurified) suspension; purification E--lipase 22.5 min, proteases 7.5 min; purification F--lipase 11 min, proteases 4 min; purification G--lipase 5.5 min, proteases 2 min; purification H--lipase 2.5 min, proteases 1 min.
⁺MPN = most probable number, here calculated as the *proportion* of viable MLM;
 Na/K = median ratio of the concentration of Na^+ ions to that of K^+ ions.

inoculation. Protocol "G" appeared optimal, in terms of preserving the viability of the organisms, while achieving adequate purification.

Development of new probes. Work with a new probe--measurement of the intracellular ratios of sodium to potassium ions by the LAMMA technique--has been continued, in collaboration with Prof. Seydel. Having established that purification protocol "G" was optimal, we applied it to suspensions of MLM harvested from mice that had been infected earlier with 10^7 organisms ip, and, after the organisms had multiplied, were administered isoniazid (INH), a drug known to be bactericidal for MLM, or other antimycobacterial drugs, the effects of which on MLM were unknown. The results of the first of these experiments, summarized in Table 2a, were disappointing. Although the LAMMA-measurements indicated both a large proportion of viable organisms in the control suspension of MLM, that had been prepared from untreated mice, and killing of a considerable number of organisms by INH, the results could not, for operational rather than theoretical reasons, be confirmed by mouse-inoculation.

Table 2. The effects of isoniazid and streptomycin on MLM, measured by titration and LAMMA

Treatment*	MPN	Median $[Na^+]/[K^+]$
Control	≥ 0.48	0.29
INH	≥ 0.0048	0.96
[SM]	≥ 0.44	0.23
SM	≥ 0.48	0.28
INH + [SM]	≥ 0.0044	0.56
INH + SM	≥ 0.0048	0.56

*Treatment was begun 157 days after inoculation of CBA mice with 10^7 MLM ip, and continued for 32 days, at which time mice were sacrificed, their spleens excised, and the MLM harvested, purified, titrated in mice for determination of the MPN, and applied to grids for measurement of the ratio $[Na^+]:[K^+]$ by LAMMA. INH was incorporated in the diet in a concentration of 0.02%. Streptomycin (SM) and SM incorporated into liposomes ([SM]) were administered ip in a dosage of 1 mg tiw.

The results of a second experiment are interesting. The effects of treatment of the mice with INH for two weeks were not overestimated; measurement of the proportion of viable MLM by titration in mice (see Table 3) demonstrated 87 per cent killing of the organisms, whereas the $[Na^+]:[K^+]$ ratio was not different from that of the MLM that had been harvested from untreated control mice. On the other hand, although measurement of the proportion of viable MLM surviving treatment with INH for four weeks was not accomplished by titration, the median $[Na^+]:[K^+]$ ratio of the INH-treated organisms was significantly larger than that of the control MLM.

The results of the most recent experiment, outlined in Table 4, are pending. Mice have been inoculated, in each case with as few as five MLM per foot pad, so that direct measurement of the proportion of viable organisms by titration in mice should be accomplished, and grids have been prepared. Until the results of this third experiment have been analysed, it appears hazardous indeed

Table 3. The effect of isoniazid on MLM, measured by titration and LAMMA

Treatment*	MPN	Median [Na ⁺]/[K ⁺]
Control--day 132	>0.48	0.51
Control--day 140	0.40	0.50
INH--17 days	0.053	0.54
Control--day 152	>0.48	0.22
INH--29 days	>0.0044	0.76

*Treatment was begun 123 days after inoculation of CBA mice with 10⁷ MLM ip. At intervals thereafter, mice were sacrificed, their spleens excised, and the MLM harvested, purified, titrated in mice for determination of the MPN, and applied to grids for measurement of the ratio [Na⁺]:[K⁺] by LAMMA. INH was incorporated in the diet in a concentration of 0.02%.

to draw conclusions. A direct, 1:1 correspondence between the [Na⁺]:[K⁺] ratio and the ability of MLM to multiply in mice has not been shown to exist. On the other hand, normal ratios and organic fragment spectra after treatment with INH for two weeks, at which time these and earlier results demonstrated impairment of viability, as measured by titration in mice, suggest that the differences of the sensitivity of the two methods may be drug-specific, as do the differences of sensitivity of the two methods to the effects of SM.

Table 4. The effects of isoniazid and clofazimine on MLM, measured by titration and LAMMA

Treatment*	MPN	[Na ⁺]/[K ⁺]
Control--day 131		
INH)		
CLOF)--15 days		
INH + CLOF)		
Control--day 147		
INH)		
CLOF)--31 days		
INH + CLOF)		
Control--day 165		
INH)		
CLOF)--49 days		
INH + CLOF)		

*Treatment was begun 116 days after inoculation of CBA mice with 10⁷ MLM ip. At intervals thereafter, mice were sacrificed, their spleens excised, and the MLM harvested, purified, titrated in mice for determination of the MPN, and applied to grids for measurement of the ratio [Na⁺]:[K⁺] by LAMMA. INH was incorporated in the diet in a concentration of 0.01%, and clofazimine (CLOF) in a concentration of 0.05%.

We plan, during the next year of this research, to begin studies with *M. leprae*. Although it is important to attempt to confirm on *M. leprae* the conclusions reached as the result of the work on MLM, one must remember that each experiment with *M. leprae* will require approximately 18 months.

Collaboration with Thailand. During the latter part of this report-period, the collaboration with Dr. Niwat Montreewasuwat has finally become a reality. Prof. Levy visited the Bangkok laboratory in February, 1989. At that time, no funds had yet been received, and preparations were being made to transfer the activities of the Raj-Pracha-Samasai Institute to a new building at some distance from the old.

Subsequent to the visit, the first funds were received, and work began in earnest. Equipment and supplies, including a laminar flow cabinet, have been purchased, and a technician has been employed and trained. The effects of purification on the viability of *M. leprae* have been studied by subjecting suspensions of the organisms to purification according to protocols C - H, and titrating the purified organisms in mice. In addition, several patients with previously untreated lepromatous leprosy have been recruited and begun on multi-drug therapy (MDT). Both before and during MDT, *M. leprae* were recovered from skin-biopsy specimens, studied with fluorescein diacetate (FDA), and titrated in mice. The results of study of the first patient, summarized in Table 5, show, at least in this one case, poor correspondence between the viability of the organisms, as measured by inoculation of mice, and intracellular esterase activity, as measured by fluorescence of FDA. The results of more recent experiments will become available only during the coming year.

Table 5. Effects of MDT on the viability of *M. leprae*

Duration of MDT (days)	Stained green by FDA (%)	MPN	% Kill
0	92.5	0.0021	---
3	70.5	0.000042	98

This work will continue, with recruitment of more patients and the addition of tellurite-reduction and LAMMA-assays of intracellular $[Na^+]:[K^+]$ ratios.