In vitro* methods for determination of viability of mycobacteria: comparison of ATP content, morphological index and FDA-EB fluorescent staining in *Mycobacterium leprae

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Summary Bacilli were purified from the 23 cases of multibacillary type of leprosy. The ATP content of these bacilli was assayed by a firefly bioluminescent technique which is capable of detecting a very small number of cultivable mycobacteria as assessed by colony counts. The ATP content was compared with morphological index (MI) and FDA-EB staining of bacilli from the same specimens. It was observed that when MI was 1% or more, the ATP content/solid bacillus was fairly constant in 15 cases studied. It ranged from 2.02×10^{-15} g to 5.60×10^{-15} g/solid bacillus (mean 3.46×10^{-15} g) and was in the same range as ATP content of viable cultivable mycobacteria. In the same 15 cases, when the green-staining bacilli was considered as 'supposedly viable bacilli', ATP content/green-staining bacillus varied up to 9-fold (0.22×10^{-15} g to 1.98×10^{-15} g/green-staining bacillus) and this did not correlate. The percentage of green-staining bacilli (FDA-EB) and solid-staining bacilli (MI) was different in all the cases. In 2 cases with 0% MI in which ATP levels were also zero, 7.5% and 21.5% green-staining bacilli were present which implies that the enzymes responsible for green-staining character may persist for some time after death. Three cases with 0% MI had also 0% green-staining bacilli and zero ATP levels, whereas in another 3 cases with zero MI significantly high levels of ATP were detected. It is inferred that solid-staining bacilli may be the viable bacilli but when MI is 0% (1% or less) sampling error or clumping may be responsible for missing out the solid bacilli in some cases. It is concluded that the ATP content of *M. leprae* appears to be an easy, rapid and sensitive tool for determining the viability for monitoring the therapy. On the other hand MI and FDA-EB staining appear to have their limitations.

Introduction

As *Mycobacterium leprae* can not be cultivated in *in vitro* systems, there is a strong need to develop other rapid screening methods for monitoring the effect of therapy as growth in the mouse footpad

takes a long time. Any reliable indirect method for determining the viability of *M. leprae* would be of great interest for assessing the progress of disease and response to therapy. The percentage of solid-staining bacilli (Morphological Index) has been used for a long time to assess the effect of therapy.^{1,2} Other studies^{3,4} postulated that nonsolid forms of bacilli were nonviable whereas elsewhere⁵ the multiplication of *M. leprae* even from specimens with 0% solid organisms was reported.

All living cells contain adenosine-5-triphosphate (ATP), which is present in fairly constant amounts in each cell type and which is rapidly lost after the cells die. These facts provide the basis of the bioluminescent assay of microbial mass which involves the measurement of light produced as a result of an enzymatic reaction catalysed by firefly luciferase. The bioluminescent assay has already been used to measure the biomass of several organisms.⁶⁻⁸ The ATP content of *M. leprae* has been measured⁹⁻¹³ and recently it has also been reported that *M. leprae* synthesizes its own ATP.¹⁴ But all these ATP measurements have been on total *M. leprae* populations and variable results have been reported.¹⁰⁻¹³ In these reports, no effort was made to estimate the number of viable cells by direct or indirect methods.

A fluorescent-staining procedure incorporating the use of fluorescein diacetate (FDA) and ethidium bromide (EB) has been recently described for *M. leprae*¹⁵ and it has been reported that there was significant decrease in the percentage of green-stained bacteria with increased periods of therapy.^{15,16} Because of varying results reported, it would be of interest to compare these methods in the same specimen from same patient. It would also be important to investigate the factors which could influence the results.

In the present study, ATP content of *M. leprae* from different multibacillary leprosy cases has been measured and its correlation with morphological index (MI) and FDA-EB fluorescent staining from the same specimen has been assessed.

Materials and methods

MATERIALS

Biopsies from 23 multibacillary leprosy cases and 5 healthy controls attending the OPD of our Institute were taken. These patients were in different stages of treatment and on different drug regimens.

Mycobacterium tuberculosis H₃₇Rv (TMC 102) and *M. lufu* (obtained from Dr F Portaels, Belgium) were grown in Sauton's medium¹⁷ and used for studying the effect of purification procedures as well as ATP extraction and assay techniques.

PROCEDURES

A Standardization of procedures

M. tuberculosis H₃₇Rv (TMC 102) and *M. lufu* were grown in Sauton's medium and log phase growth were harvested. Suspensions were prepared and used for studying the effect of purification and extraction procedures. The purification procedure described by Dhople & Storrs¹⁰ was used. For decontamination 2% (0.5 M) and 4% (1 M) NaOH was used with 20, 30 and 60 min of incubation period at room temperature. For ATP extraction, (i) trichloroacetic acid (TCA) extraction technique,^{18,19} (ii) tris boiling method,^{18,19} and (iii) chloroform boiling method²⁰ were compared.

For estimating the correlation between ATP content and viable numbers, the mycobacterial suspensions were diluted in buffered Tween and were plated on Lowenstein Jensen medium.

B Processing of biopsies

(i) Biopsies were homogenized as described in another study.¹⁰

(ii) The suspensions after following the modified decontamination procedure,¹⁰ were used for ATP extraction. From the final suspensions, duplicate smears were prepared on circular slides and processed as described in steps (iii) and (iv). In the decontamination procedure 2% NaOH (0.5 M) finally was used as this gave optimum results. ATP was extracted by the Tris-EDTA boiling method as this method gave the optimum and reproducible results in our experimental system.

(iii) From the suspensions, the duplicate smears were prepared on circular slides and stained with Ziehl-Nielsen staining and counting was done by the method of McRae & Shepard.²¹ Morphological Index (MI) was calculated on these smears by the technique of McRae & Shepard.²¹ From the total counts and % solid-staining bacilli (MI), the population of solid bacilli was calculated.

(iv) The other set of smears was stained with fluorescein diacetate (FDA) and ethidium bromide (EB) and examined under the fluorescent microscope by the technique of Kvach *et al.*¹⁵ From the total counts and % green-staining bacilli, the green-staining bacillary population was calculated.

(v) ATP was assayed in a Lumitran L-3000 ATP Photometer (New Brunswick Inc. USA) by using the method of Lehtokari *et al.*¹⁹ One minute integrated ATP counts were taken and concentration were calculated using the standard ATP from LKB.

(vi) After measuring the ATP content in the total bacillary population, ATP content/solid bacillus and ATP content/green-staining bacillus were calculated for each sample as following:

$$\text{ATP content/solid bacillus} = \frac{\text{Total ATP content}}{\text{Number of solid-staining bacilli estimated in step (iii)}}$$

$$\text{ATP content/green staining bacillus} = \frac{\text{Total ATP content}}{\text{Number of green-staining bacilli estimated in step (iv)}}$$

Results

The results are detailed in Tables 1–4.

CORRELATION BETWEEN VIABLE COLONY COUNTS/ATP CONTENT

The viable number of bacilli were determined by colony counts after diluting the suspension in buffered Tween and these correlated directly with ATP levels in *M. tuberculosis* and *M. lufu*. Using this procedure, it was possible to detect < 100 viable mycobacteria. This ATP content was found to be reproducible in 3 sets of observations even with 10–100 viable organisms. The ATP content/viable cell was found to be: $2.0\text{--}3.4 \times 10^{-15}$ g (*M. tuberculosis*) and $3.5\text{--}5.8 \times 10^{-15}$ g (*M. lufu*) respectively.

EFFECT OF PURIFICATION PROCEDURE

Except for prolonged incubation with 4% NaOH (1 M), no other steps in the purification procedure of Dhople & Storrs¹⁰ were found to interfere with the ATP levels in *M. tuberculosis*, *M. lufu* and human-derived *M. leprae*. The results are detailed in Table 1. It was found that ATP levels significantly fell down when the incubation with 4% (1 M) NaOH was done for 30 min or more whereas 2% NaOH (0.5 M) did not affect the mycobacterial ATP even after 1 hr of treatment. This 2% (0.5 M) NaOH treatment was sufficient to remove the host derived ATP completely. Based on these observations we have used 0.5 M NaOH in our study.

Table 1. Effect of 4% (1 M) NaOH and 2% NaOH (0.5 M) on ATP levels in different mycobacteria. ATP levels (g)*/ 10^6 bacilli

	Time of incubation			
	0 h	20 min	30 min	1 h
4% (1 M) NaOH				
<i>M. tuberculosis</i>	2.60×10^{-9}	2.85×10^{-9}	1.57×10^{-9}	0.91×10^{-9}
<i>M. lufu</i>	4.27×10^{-9}	3.94×10^{-9}	2.99×10^{-9}	0.85×10^{-9}
<i>M. leprae</i>	1.65×10^{-10}	1.55×10^{-10}	1.07×10^{-10}	0.53×10^{-10}
2% (0.5 M) NaOH				
<i>M. tuberculosis</i>	3.10×10^{-9}	2.85×10^{-9}	3.00×10^{-9}	2.90×10^{-9}
<i>M. lufu</i>	5.30×10^{-9}	4.80×10^{-9}	5.10×10^{-9}	4.90×10^{-9}
<i>M. leprae</i>	2.50×10^{-10}	2.70×10^{-10}	2.40×10^{-10}	2.25×10^{-10}

* Mean: based on 3 observations.

ATP EXTRACTION PROCEDURES

We have compared TCA extraction method, chloroform heat method and Tris-EDTA boiling method for extraction of ATP from *M. tuberculosis*, *M. lufu* and human-derived *M. leprae*. It was observed that TCA extraction method extracted the maximum levels of ATP from each of the mycobacteria tested. Considering it was (100%), the efficiency of other methods was calculated. Finally Tris-EDTA boiling method was used in our study because of its convenience and optimum results. The results are detailed in Table 2.

Table 2. Efficiency (%) of different methods for ATP extraction based on two observations

	<i>M. tuberculosis</i>	<i>M. lufu</i>	<i>M. leprae</i>
TCA extraction method	100	100	100
Chloroform-heat method	86	80	76
Tris-EDTA boiling method	98	96	98

ATP LEVELS, MI AND FDA-EB STAINING OF MATERIAL PROCESSED FROM BIOPSIES

The results are summarized in Tables 3 and 4.

It was observed that: (i) MI of patients who were in different stages of therapy varied from 0 to 9%; (ii) the percentage of green-staining bacilli by FDA-EB staining in these smears varied from 0 to 45%; (iii) the percentage of green-staining bacilli and solid-staining bacilli (MI) were different in all cases. Not only were the absolute values different, but there was no apparent relationship between MI and percentage of green-staining bacilli; (iv) the host ATP in the skin tissues could be completely removed by the purification procedure used in the study; (v) when the MI was 1% or more, the ATP content per solid bacillus was fairly constant in 15 of the cases studied. It ranged from 2.02×10^{-15} g to 5.60×10^{-15} g/solid bacillus with mean 3.46×10^{-15} g; (vi) when the green-staining bacilli were considered as 'Supposedly viable bacilli', the ATP content/green-staining bacillus from different multi-bacillary cases showed huge variations. The ATP content/green-staining bacillus ranged from 0.22×10^{-15} g to 1.98×10^{-15} g among different cases. However in 9/15 cases, the ATP content/green-staining bacillus ranged from 0.22×10^{-15} g to 0.66×10^{-15} g; (vii) three cases with 0% MI,

Table 3. The ATP content per solid-staining bacillus and per green-staining bacillus in multibacillary patients

Patient	MI (%)	Green-staining by FDA-EB (%)	ATP content/solid bacillus* (g)	ATP content/green-staining bacillus† (g)
1 RB	9.0	38.0	3.80×10^{-15}	0.92×10^{-15}
2 AC	8.0	45.0	4.48×10^{-15}	0.89×10^{-15}
3 BH	5.5	20.0	5.60×10^{-15}	1.33×10^{-15}
4 BS	5.0	33.5	3.81×10^{-15}	0.66×10^{-15}
5 AF	5.0	18.0	3.60×10^{-15}	1.05×10^{-15}
6 BS	4.5	24.5	2.24×10^{-15}	0.44×10^{-15}
7 KC	4.5	13.0	5.38×10^{-15}	1.98×10^{-15}
8 IP	5.0	15.0	2.60×10^{-15}	0.62×10^{-15}
9 MH	4.0	30.0	2.10×10^{-15}	0.22×10^{-15}
10 NZ	3.5	21.5	2.24×10^{-15}	0.44×10^{-15}
11 LA	3.0	19.5	3.90×10^{-15}	0.66×10^{-15}
12 AM	3.0	10.0	3.58×10^{-15}	1.10×10^{-15}
13 KA	2.5	14.0	2.69×10^{-15}	0.44×10^{-15}
14 KC	2.5	22.0	2.02×10^{-15}	0.22×10^{-15}
15 PD	1.0	15.0	3.90×10^{-15}	0.22×10^{-15}

* Mean, 3.4×10^{-15} g/solid bacillus.† Mean, 0.8×10^{-15} g/green-staining bacillus.**Table 4.** ATP content and percentage of green-staining bacilli in patients who were having 0% MI*

Slide No.	Patient	MI (%)	% green-staining bacilli (%)	ATP content/bacilli†
1	BU	0.0	0.0	$0/1.8 \times 10^5$
2	GR	0.0	0.0	$0/1.5 \times 10^7$
3	PR	0.0	0.0	$0/0.9 \times 10^5$
4	GA	0.0	7.5	$0/11.6 \times 10^6$
5	MH	0.0	1.5	$0/1.8 \times 10^5$
6	RA	0.0	6.5	1.7×10^{-13} g/ 2.6×10^6
7	SL	0.0	5.0	2.8×10^{-13} g/ 6.8×10^5
8‡	TA	0.0	7.5	5.6×10^{-12} g/ 6.3×10^5

* Mean, biopsies at serial Nos 1–7 were negative for growth in a normal mouse footpad.

† Mean, total bacilli.

‡ Mean, results of mouse footpad inoculation not available.

also had 0% green-staining bacilli and zero ATP levels (Table 4); (viii) in the cases with 0% MI, ATP levels were also zero in 5 cases. In 3 other cases with zero MI very high levels of ATP were detected (Table 4); (ix) in 2 cases with 0% MI, in which the ATP levels were also zero, 7.5% and 21.5% green-staining bacilli were present in the smears (Table 4).

Discussion

ATP content of *M. leprae* has been measured and reported previously.^{9–12} Similarly the ATP content

of cultivable mycobacteria has been reported earlier.^{10,11,12} We have observed in this study, that ATP content of cultivable mycobacteria directly correlates with viable counts and it is a sensitive, stable, constant index of viability. In the present study it has also been observed that when the 'solid-staining bacillus' is supposed as a viable bacillus, its ATP content is reasonably constant and then this ATP content is in the same range as other viable cultivable mycobacteria studied by us and reported previously.¹¹ ATP content of *M. leprae* as observed by us and also reported by others¹¹⁻¹³ is higher than the levels reported in other studies.^{9,10} This could be due to technical differences, e.g. purification and extraction procedures etc. as seen in the present study. It has been reported previously that ATP content of *M. leprae* correlates with viability in mouse foodpad.²³ In the present study, ATP content has been found to be a stable index of viability in cultivable mycobacteria as determined by colony counts and solid-staining bacillus in *M. leprae* has the similar ATP content in 15 different cases when this is supposed as index of viability. It may thus be inferred that solid-staining bacilli are the viable bacilli as postulated by others.^{4,24,25}

The calculation of MI appears to have practical difficulties. The growth in mouse foodpad has been reported even in cases with zero MI.⁵ Also in the present study in 3 of the 8 cases with zero MI, very high ATP levels were detected, which means that there were a significant number of viable bacilli in these specimens. These observations could mean that while the solid-staining bacilli may indeed be the 'viable bacilli' as postulated earlier, yet the MI as *per se* is a poor index of viability as seen in another study⁵ and also as observed in the present study. It is likely that due to sampling of a few hundred bacilli and clumping, the solid bacilli are missed resulting in false zero readings. Though the MI may have a limited role in determining the effect of therapy it does not appear to be a reliable index of viability for monitoring the therapy especially when 0% values are recorded. Thus the important limitation of MI is its low sensitivity even though it appears to be a good measure of viability. On the other hand, the ATP content of *M. leprae* populations especially when it is decreasing or has reached zero, can be used as a reliable index of determining the viability even when the viable mycobacteria are very few. Thus it will be a sensitive tool for determining the viability of *M. leprae* from clinical specimens particularly for monitoring the effect of therapy.

Apparently the MI and green-staining populations by FDA-EB staining detect different populations as seen in the present study and as reported by another study.¹⁶ The figures for both these indices from the same specimen are significantly different (Table 3). It has been reported that the percentage of green-staining bacilli decreases with treatment.^{15,16} If the green-staining bacilli are taken as 'index of viability', no correlation in the ATP content of green-staining bacilli from different cases was observed. ATP content/green-staining bacillus varied up to 9-fold in the 15 cases in which ATP content/solid bacillus ranged from 2.02 to 5.6×10^{-15} g (approximately a 2.8-fold variation). FDA-EB staining may have some role in monitoring the effect of therapy like MI, but it appears that it may not directly correlate with viability. In 2 cases the ATP levels were zero yet significant percentage of green-staining bacilli were present. It appears that the enzymes responsible for the green-staining character by FDA-EB staining take some time to degenerate after death whereas ATP decays very fast and thus will correlate better with viability as reported earlier²³ and as observed by us in case of cultivable mycobacteria. It may be mentioned that 7/8 cases with 0% MI in the present study were negative for growth in normal mouse footpad. Two of them who were negative in mouse footpad had significantly high ATP levels (Table 4). It would be of interest to follow patients on MDT and compare these parameters with multiplication in the immunosuppressed experimental hosts as normal mouse may miss small numbers of viable organisms as reported elsewhere²⁶ and as seen in the present study.

It is concluded that ATP content of *M. leprae* from leprosy cases appears to be a easy, rapid tool for determining the viability and thus can be a more sensitive tool for monitoring the effect of therapy than the conventional MI and FDA-EB staining which appear to have their limitations.

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NEWS AND NOTES

Small grants programme for development of appropriate technology

Grants for work on assays to diagnose diseases prevalent in developing countries are now available through a project known as DiaTech (Diagnostic Technology for Community Health). The grants, awarded to qualified investigators at public and private institutions (both profit and non-profit) around the world, will support various research-related activities dealing with these diagnostic assays—including development of reagents, test kit design, field evaluation, personnel training, manufacture, introduction, distribution, and impact evaluation. DiaTech can cofund projects with other granting agencies. All of the awards, which carry stipends ranging up to US\$300,000 over a two-year period, are subject to peer review.

The DiaTech project is administered by the Program for Appropriate Technology in Health (PATH), a nongovernmental non-profit organization, under a cooperative agreement with the United States Agency for International Development (AID).

DiaTech is managed both by PATH personnel and by additional professional staff members from Johns Hopkins University, the University of Maryland, the Thai Red Cross Society, and other institutions. A technical advisory group composed of distinguished scientists evaluates research and development proposals for funding and monitors work in progress.

The DiaTech project's basic aim is to better the health of people in developing countries through improvement of available and appropriate assays for diagnosing infectious diseases.

Further information about DiaTech grants, including specific guidelines for preparing proposals, may be obtained by writing or calling the Program Administrator/DiaTech, PATH, 4 Nickerson Street, Seattle, Washington 98109-1699, USA (Tel. 206-285-3500).

New joint funding venture: UNDP/World Bank/TDR

The UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and the Rockefeller Foundation (RF) announce a new joint funding venture to promote laboratory, clinical and field research on major tropical diseases in countries where these diseases are endemic. The purpose of this venture is to involve and link institutions of quality in the application of recent advances and knowledge in biomedical sciences, epidemiology and social sciences to the development, testing and application of new ways of preventing, treating and controlling these diseases.

Partners in research and training

To achieve this goal, TDR and RF will support the establishment of partnerships between two or more groups and/or institutions that can, by pooling their resources, provide both the expertise and facilities required as well as the unique research context and field research opportunities which exist in endemic areas. Partnerships are envisaged between groups that together are equipped to apply modern science and technology to these problems and have demonstrated potential to assimilate and apply these technologies in vulnerable target populations in tropical disease-endemic countries.

How to apply

Applications are invited from research groups and/or institutions that have identified one or more counterpart groups and/or institutions with whom they currently have or have had contacts. One or more of the partners must be located in a local institution in a tropical disease-endemic country. The partner institutions should submit jointly a letter of intent, not exceeding three pages, which should: describe the goals of joint activities and the unique contribution and resources of each partner; outline the proposed research and research training activities; briefly summarize previous experiences; and indicate the nature and estimated cost per partner of the total support requested. Letters of intent must be received by no later than 1 December 1987.

Partner institutions that are selected, in December 1987 by a committee of experts, as candidates for support will be requested to submit a detailed proposal, not exceeding 15 pages, by 1 March 1988. Instructions for the final proposal will be provided upon notification of selection. Funds awarded for this joint venture will be based on comparative costs of research and cooperative activities and are expected to be in the range of US\$ 40,000—120,000 per partner per year, for a period of up to five years, subject to annual review of progress.

Where to apply

All letters of intent should be sent to *both* funding agencies, one copy addressed to Director, TDR, and one copy to the Director of Health Sciences, RF, at the addresses given below:

Director, UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR), World Health Organization, 1211 Geneva 27, Switzerland or Director of Health Sciences, 1133 Avenue of the Americas, New York, N.Y. 10036, United States of America.