INTERNATIONAL JOURNAL OF LEPROSY

Volume 53, Number 3
Printed in the U.S.A.
(ISSN 0148-916X)

INTERNATIONAL JOURNAL OF LEPROSY
And Other Mycobacterial Diseases

VOLUME 53, NUMBER 3 SEPTEMBER 1985

Results from Cation and Mass Fingerprint Analysis of Single Cells and from ATP Measurements of *M. leprae* for Drug Sensitivity Testing: A Comparison[1]

U. Seydel, B. Lindner, and A. M. Dhople[2]

Many problems in the treatment of leprosy arise from the inability to cultivate *Mycobacterium leprae in vitro*. Information on the influence of a given therapy on the bacterial load of a patient—as, for instance, on alterations of the physiological state of the bacteria, on the possible existence or development of a drug resistance, and on the "persister" hypothesis—cannot, therefore, be obtained from standard bacteriological test assays. The only method presently available to determine the viability and growth of *M. leprae* is the mouse foot pad test ([12]), a procedure which is not only rather time consuming but also expensive and somewhat difficult.

To overcome these problems, efforts have been focused on the development of alternative techniques in recent years. Dhople and co-workers have employed adenosine triphosphate (ATP)—determined as an average value from 10^6 cells—as a biochemical indicator in chemotherapeutic studies on the status of *M. leprae* harvested from patients ([1, 3]). Another approach was started about three years ago by Seydel and Lindner utilizing the mass spectrometric analysis of single *M. leprae* cells, which became feasible through the development of the laser microprobe mass analysis (LAMMA). It could be shown with cultivable strains that the intracellular ratio of sodium and potassium cations (NA^+,K^+-ratio) is a sensitive indicator of alterations in the physiological state of the cells. Measurements from a larger number ($\approx$500) of single cells allow the calculation of distribution patterns of this ratio. These patterns provide information on the drug response of the bacterial population which is beyond the scope of established microbiological methods ([7, 10, 11]).

In addition to the cation signals, the single cell mass spectra contain mass peaks which originate from the organic matrix. Due to the complexity of this matrix and to the yet unknown fragmentation mechanisms during ion formation, an evaluation of the in-

[1] Received for publication on 13 November 1984; accepted for publication in revised form on 18 March 1985.

[2] U. Seydel, Dr.rer.nat.habil., Dipl.Phys.; B. Lindner, Dr.rer.nat., Dipl.Phys., Division of Biophysics, Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, D-2061 Borstel, Federal Republic of Germany. A. M. Dhople, Ph.D., Medical Research Institute, Florida Institute of Technology, 3325 W. New Haven Avenue, Melbourne, Florida 32901, U.S.A.

formation contained in this "organic pattern" (mass fingerprints) can be performed only by applying multivariate analysis procedures ([8, 9]).

In this paper, results for the intracellular Na^+,K^+-ratio from single cell mass analyses of bacteria isolated from 31 leprosy patients before and/or after treatment with 4,4'-diaminodiphenyl sulfone (dapsone, DDS) are compared with those from the respective ATP measurements and from the mouse foot pad tests. Also, the first results on the fingerprint evaluation of this patient pool are presented.

MATERIALS AND METHODS

Patients. A total of 31 patients were selected randomly from Bombay and Surinam. Six of these patients had already been under DDS monotherapy when they entered the study. Skin biopsies were obtained from the other 25 patients before and 4 or 10 months after beginning DDS treatment. Biopsy specimens (±5 mm) from each patient were stored at −76°C and then shipped to the Medical Research Institute, Florida Institute of Technology, Melbourne, Florida, U.S.A.

Suspensions of *M. leprae*. The method described earlier ([4]) was adopted for the separation and purification of *M. leprae*. In short, it consisted of the following steps: homogenization in 0.5 M phosphate buffer, pH 7.0; decontamination with 4% NaOH followed by neutralization with 1 N HCl; treatment with 0.1% each of trypsin, chymotrypsin, and collagenase; exposure to Triton X-100 (final concentration 0.1%) followed by ATPase (final concentration 0.17%, containing 0.005 M $CaCl_2$).

ATP assay. The purified suspension in 0.05 M Tris buffer, pH 7.7, was then taken for ATP extraction by the method of Dhople and Hanks, using chloroform and heat ([2]). The final extract was suspended in Tris buffer, and 0.1 ml of the extract was then injected into 0.1 ml of a luciferase-luciferin system (DuPont Company, Wilmington, Delaware, U.S.A.) supplemented with pure luciferin (Sigma Chemical Company, St. Louis, Missouri, U.S.A.). The peak height of the reaction was measured on a Chem-Glow Photometer (American Instrument Company, Silver Spring, Maryland, U.S.A.) to calculate the ATP content of the *M. leprae*.

Bacterial counts. The pinhead method of Hanks, *et al.* ([5]) was used to enumerate *M. leprae* in purified suspension.

Mouse foot pad assay. The procedure of Shepard ([12]) was adopted for these assays; 0.03 ml of bacterial suspension containing 5×10^3 *M. leprae* was inoculated into the hind foot pads of three-month-old BALB/c female mice. The mice were fed powdered laboratory chow alone or chow mixed with dapsone, 0.0001%, 0.001%, or 0.01% (w/w). Eight months after inoculations, the mice were killed, and the foot pad tissues removed and homogenized in balanced salt solution containing 0.1% bovine serum albumin using a Ten Broeck glass homogenizer. The *M. leprae* in the suspensions were then enumerated using the pinhead method ([5]).

Laser microprobe mass analysis (LAMMA). This technique has been described in detail elsewhere ([6]). Briefly, in the LAMMA instrument a high-energy, ultraviolet laser pulse is focused through the objective of a light microscope onto the sample to be analyzed. The laser pulse evaporates and partly ionizes the sample at a lateral resolution down to approximately 1 μm, dependent on sample conditions and on laser energy. The positive and negative atomic and molecular (fragment) ions produced are registered alternately by means of a time-of-flight mass spectrometer at a mass resolution of about 800. The detection limits for sodium and potassium go down to about 10^{-20} g in an analyzed volume of 1 μm^3.

Sample preparation for LAMMA. Parts of the purified suspensions used for the ATP assay were washed and centrifuged (at $2000 \times g$ for 10 min each) twice in distilled water. The last sediment was resuspended in distilled water and one drop of this suspension was transferred to a Formvar-coated copper grid (as used in electron microscopy), and excess fluid was drained off with tissue. In this way, a widespread distribution of the bacteria was achieved, allowing the laser vaporation of one single cell at a time.

Single cell mass analysis. The intracellular Na^+,K^+-ratio was determined from the intensities of the $^{23}Na^+$- and $^{39}K^+$-mass

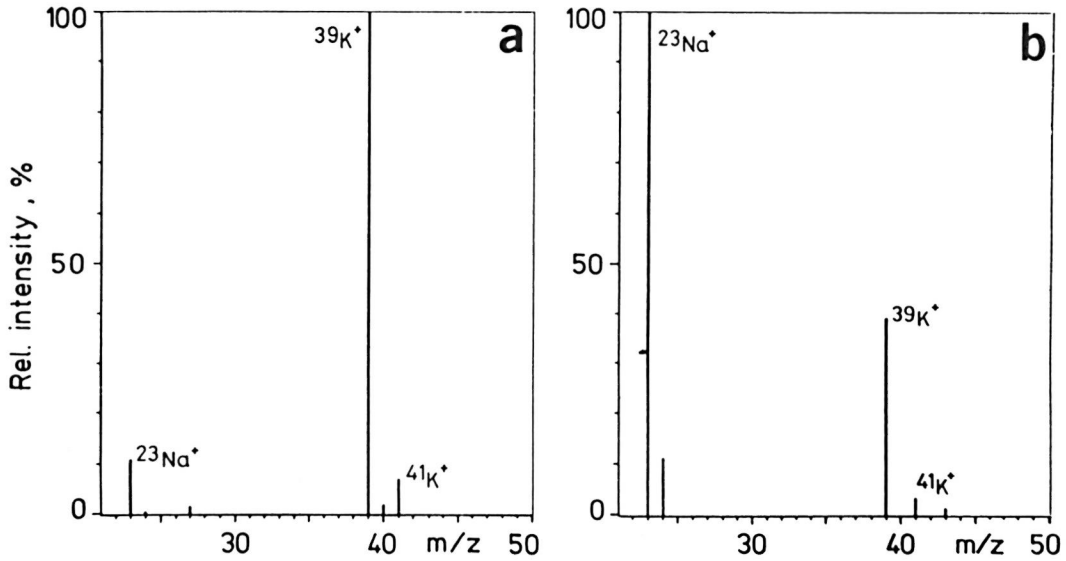

FIG. 1. Cation signals in mass spectra obtained from one single cell, in each case, isolated from an untreated (a) and from a DDS-treated (b) patient, respectively.

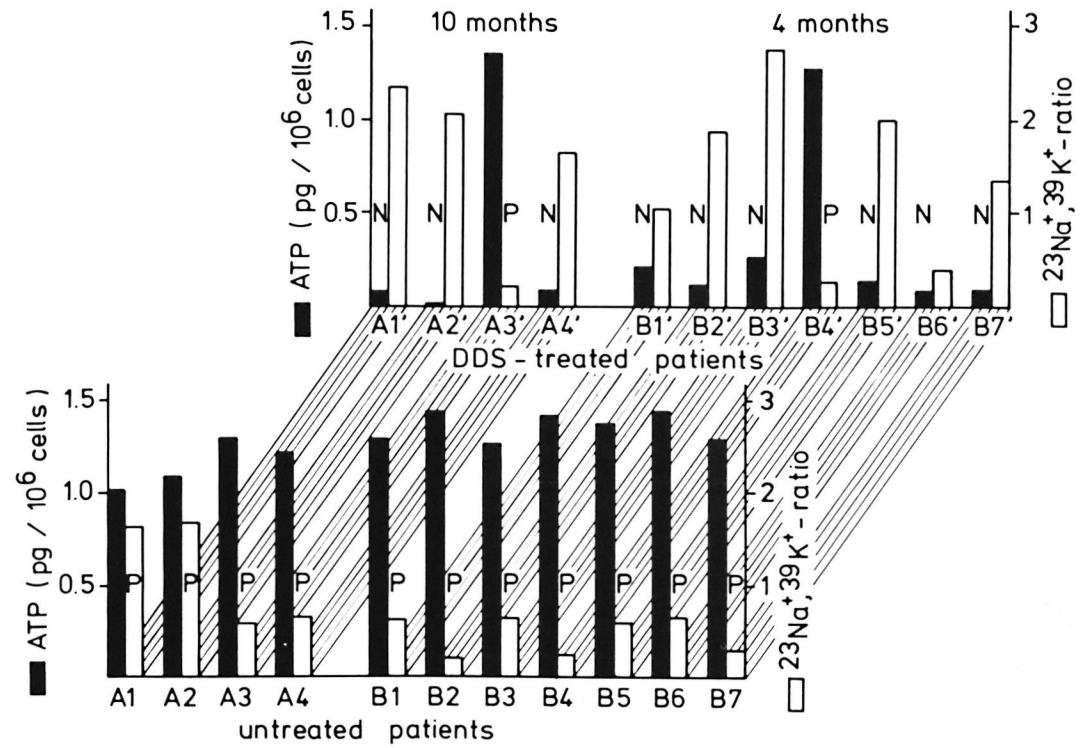

FIG. 2. Comparision of ATP values, $^{23}Na^+$,$^{39}K^+$-ratios, and mouse foot pad test results for *M. leprae* samples isolated from 10 patients before and 4 or 10 months after the initiation of DDS monotherapy. P and N indicate positive or negative results from the mouse foot pad assay, respectively.

peaks as an average of 200 single cell measurements. (It should be mentioned that this ratio does not necessarily reveal the true intracellular ratio because of different ionization efficiencies for the two cations.) For mass fingerprinting, 120 spectra in the mass range m/z 45 to 250 were registered from each sample and evaluated after a multidimensional scaling procedure described elsewhere ([8]).

RESULTS

The ATP bioluminescence assay, its application to *M. leprae,* and its comparison with the mouse foot pad test have been discussed comprehensively elsewhere ([1, 3]). Here, the results from the mass spectrometric determination of the intracellular Na^+,K^+-ratio of single *M. leprae* cells are compared with those from the ATP assay and—in a follow-up study (Fig. 2)—also with those from the mouse foot pad assay.

Figure 1 shows the Na^+ and K^+ signals obtained from single *M. leprae* cells isolated from an untreated (a) and a DDS-treated (b) patient, clearly demonstrating a shift in the intracellular concentration of these cations after only four months of treatment.

In Figure 2, the ATP values and the $^{23}Na^+$,$^{39}K^+$-ratios are given for bacilli from 11 patients before treatment and after 4–10 months of DDS monotherapy. Bacteria from untreated patients should have a high ATP content and a low Na^+,K^+-ratio. Successfully treated (impaired) cells should, in contrast, have low ATP signals and high cation ratios. Out of the total of 22 comparative measurements, the results on 3 patients (A1, A2, B6′) do not agree for the two methods applied. For patients A1 and A2 a sodium contamination of the samples could explain the high Na^+,K^+-ratios, while for patient B6′ it cannot be decided definitely which of the two methods did not give reliable results. The mouse foot pad assays on the biopsies from these patients are designated with "p" (positive) or "n" (negative). Positive results indicate that *M. leprae* from these specimens gave standard growth curves ($1.85–5.22 \times 10^6$ *M. leprae* per foot pad) in the foot pads of mice eight months after inoculation. Negative results indicate the inability of those *M. leprae* to multiply in the foot pads of mice. Furthermore, *M. leprae* from patients A3 and B4 multiplied in the foot pads of mice receiving 0.0001% dapsone mixed in their food. Thus, excellent correlations are seen among ATP assays, mouse foot pad assays, and Na^+,K^+-ratios.

Interestingly, both techniques indicated that the two patients A3 and B4 did not show a significant response to the DDS treatment (A3′, B4′), which might be an early indication of dapsone resistance. This was, in fact, subsequently confirmed with mouse foot pad assay results. This observation will be referred to later.

Figure 3 presents a correlation between the two methods for *in vitro* viability determination for all 31 patients. The points form two clearly separated clusters which can be assigned to viable and impaired *M. leprae* cells, respectively. Again here, the points corresponding to the three patients A1, A2, and B6′ are outliers. The cluster assigned to the impaired cells shows a larger

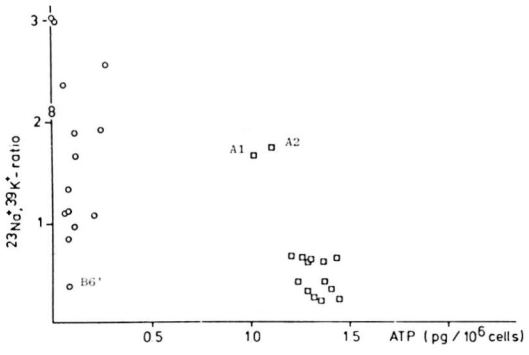

FIG. 3. Correlation between the ATP values and the cation ratios for all 31 samples investigated (□ = unimpaired, O = impaired cells). Points A1, A2, and B6′ refer to the corresponding patients in Figure 2.

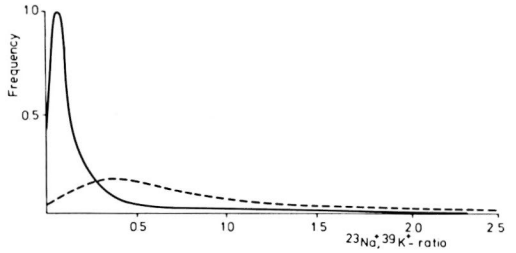

FIG. 4. Distribution of the intracellular $^{23}Na^+$,$^{39}K^+$-ratios for two *M. leprae* populations of 400 cells each isolated from the same patient before (——) and 4 months after (-----) the beginning of DDS therapy.

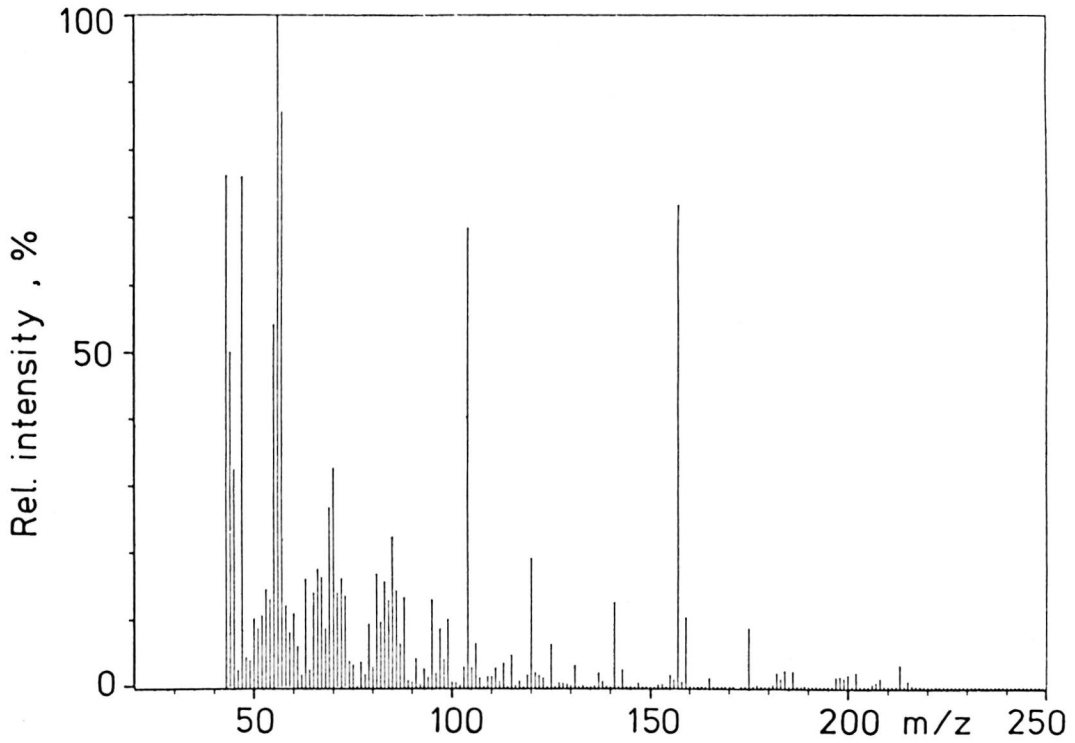

FIG. 5. Mass spectrum—an average of over 120 single cells mass analyses (from patient B2 of Fig. 2)—as an example for an "organic" mass fingerprint.

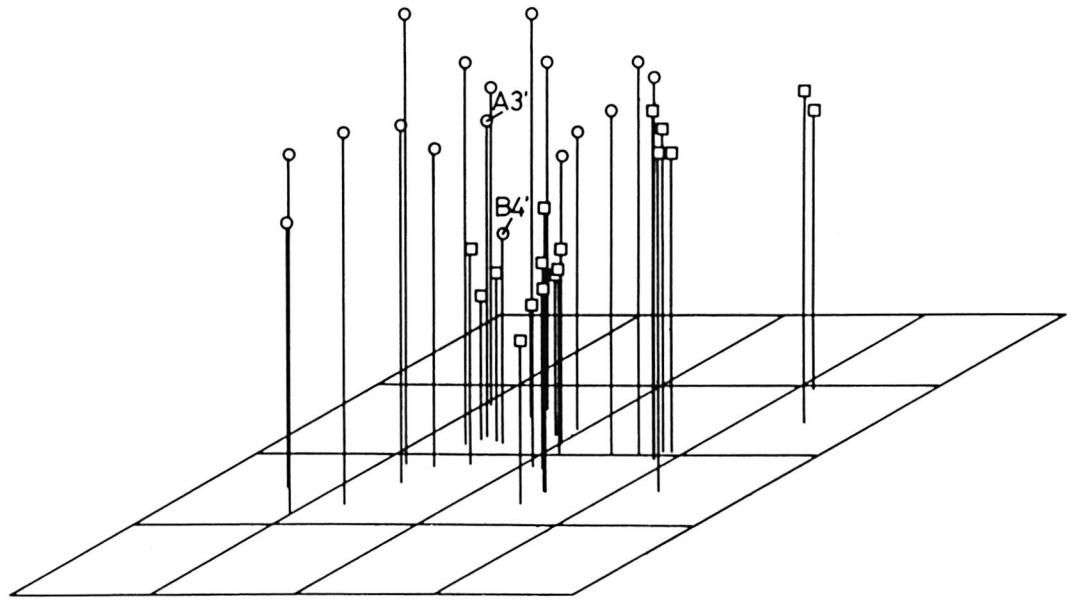

FIG. 6. Three-dimensional nonlinear map of the similarity relationships among all 31 samples under investigation. Each point represents an averaged fingerprint of over 120 single cell mass analyses (□ = fingerprints from cells of untreated patients; ○ = DDS-treated patients). Points A3′ and B4′ refer to the corresponding patients in Figure 2.

spread in the cation ratios than in the ATP contents. The lower spread in the ATP values might be due to the fact that the ATP content of the impaired cells is too low to be quantitatively measured by the bioluminescence assay from a total of only 10^6 cells. Under the conditions adopted in the present studies, the lower limit of the ATP assay is 0.2 picograms. Thus, taking 3×10^6 cells per assay, it has been possible to quantitatively detect up to 95% loss of original ATP from the cells from treated patients.

In Figure 4, the distributions for the intracellular Na^+,K^+-ratios as measured from 400 single cells in each case are plotted for a patient before and after 4 months of DDS treatment. Besides a marked shift of the median to higher values, the distribution for the treated cells shows a significant broadening. This reflects the large band width in the physiological state of the bacteria of the treated sample due to variations in the drug response of the individual cell.

In Figure 5, a mass spectrum—an average of over 120 single cell mass analyses (from patient B2 of Fig. 2)—is given as an example of an "organic" mass fingerprint. The four mass peaks at m/z 104, 120, 141, and 157 are characteristic for the genus *Mycobacterium.*

In Figure 6, the evaluation of 31 such fingerprints on the basis of statistically significant differences in the mass peaks of organic origin between untreated and treated *M. leprae* cells is given. The three-dimensional plot is a nonlinear map of the similarity relations among all samples under investigation. Each point represents the average of over 120 single cell mass spectra obtained from one sample. These averaged spectra are separated into two groups, untreated and DDS treated, respectively. The measuring points corresponding to the two patients not responding to DDS treatment (A3′ and B4′ in Fig. 2) lie, in one case, clearly within the region of the DDS-treated samples and in the other case, on the border between treated and untreated samples.

DISCUSSION

The advantages of the ATP assay over the mouse foot pad test have been discussed in previous papers ([1, 3, 4]). They are: fast availability and reliability of results, low cost, and higher convenience. What are now the advantages of the single cell mass analysis as compared to either one of these methods or to both of them? The availability of the results from LAMMA is as fast and the results from the average cation ratios are as informative as those from the ATP assay. The LAMMA instrument itself is extremely expensive, whereas the costs for the experiments for the described purposes are very low. It is reasonable to assume that the single cell technique will find its place in research rather than in field studies, especially when considering the possibility of getting additional and more detailed information from this method. Since the cation ratios are measured from single cells, the distribution patterns within a population reflect the diversity of drug responses of the individual cells. From the shape of these distributions, conclusions may possibly be drawn on the mode of action of a particular drug. Furthermore, it can be expected that a correlation between the morphologic structure and the physiological condition of an individual cell will be achieved. This could provide a more reliable basis for a purely microscopic evaluation of biopsies in field studies. The example of a fingerprint evaluation does not render any information beyond that available from the cation measurements. However, from the location of a particular measuring point in the presentation of similar relationships together with the value of the corresponding Na^+,K^+-ratio, conclusions can be reached on the physiological condition of the bacilli from a patient. Hopefully, these techniques eventually might allow conclusions to be reached on an existing or a developing drug resistance without knowledge of a patient's history (e.g., drug ingestion).

For quantitative statements, of course, more data have to be acquired and the evaluation procedure has to be improved. Although the presented results give rise to some optimism, these demands are also valid for a conclusive evaluation of the reliability of these single cell mass spectrometric investigations.

SUMMARY

The physiologic states of *Mycobacterium leprae* isolated from patient biopsies were studied using single cell mass spectrometry

by laser microprobe mass analysis (LAMMA) and ATP bioluminescence assay. The changes in the physiologic state of *M. leprae* after the patients had been treated with dapsone (DDS) monotherapy were also studied. The shift of the low intracellular Na^+,K^+-ratio of untreated *M. leprae* cells to higher values under DDS therapy, as measured from a limited number of single bacteria, correlates with a decrease in the ATP content. Further information on the influence of the drug could be drawn from the multivariate analysis of mass fingerprints of the organic matrix of the cells. Evidence is provided that the combination of the measurement of the intracellular cation ratios and of the mass fingerprint analysis could give fast answers to the question of drug resistance and to the persister hypothesis. The ATP bioluminescence assay and the single cell mass analysis should be alternatives to the mouse foot pad test.

RESUMEN

Se estudiaron los estados fisiológicos de *Mycobacterium leprae* aislados de biopsias de pacientes, usando espectrometría de masas con rayo laser (LAMMA) y el ensayo de bioluminiscencia del ATP.También se estudiaron los cambios en el estado fisiológico del *M. leprae* después del tratamiento con dapsona (DDS). El cambio de una baja relación de Na^+/K^+ intracelular (en las células de *M. leprae* de pacientes sin tratamiento) a valores más elevados (en células de *M. leprae* de pacientes tratados) correlacionó con una disminución en su contenido de ATP. Del análisis de los espectros de masas correspondientes a la matríz orgánica de las células, se puede obtener información adicional sobre la influencia de la droga. Se proporcionan evidencias de que combinando la medición de la relación de cationes intracelulares y el análisis de los espectros de masas, se pueden dar respuestas rápidas a la pregunta sobre resistencia a la droga y a la hipótesis de la persistencia. El ensayo de la bioluminiscencia del ATP y el análisis de masas en células aisladas deben ser métodos alternativos de la clásica prueba del cojinete plantar del ratón.

RÉSUMÉ

On a étudié les caractéristiques physiologiques de *Mycobacterium leprae,* recueillies dans des biopsies de malades, en utilisant une méthode de spectrométrie de masse sur cellule unique par microsonde au laser—laser microprobe mass analysis (LAMMA)—et par bioluminescence de l'ATP. On a également étudié les modifications survenues dans la physiologie de *M. leprae* après que les malades aient été traités par la monothérapie à la dapsone (DDS). La conversion d'un rapport peu élevé Na^+/K^+ observée dans des cellules de *M. leprae* non traitées, en des valeurs plus élevées après thérapeutique par la DDS, mesurée dans un nombre limité de bactéries isolées, présentait une corrélation avec la diminution du contenu en ATP. On peut tirer des informations supplémentaires concernant l'influence du médicament, de l'analyse multivariée des empreintes de masse sur la matrice organique des cellules. Il apparaît dès lors que la combinaison de la mesure du rapport des cations intracellulaires et de l'analyse des empreintes de masse (mass fingerprint analysis) pourraient fournir des réponses rapides quant à la question de la résistance médicamenteuse et à l'hypothèse des bacilles persistants. Les essais recourant à la bioluminescence de l'ATP, de même que l'analyse de masse de cellules isolées pourraient remplacer les épreuves d'inoculation dans le coussinet plantaire de la souris.

Acknowledgments. This investigation received financial support from the Deutsches Aussätzigen-Hilfswerk—DAHW (German Leprosy Relief Association). The authors are grateful to Ms. Anna Grete Dethlefs for performing the LAMMA measurements.

REFERENCES

1. Dhople, A. Adenosine triphosphate content of *M. leprae* from leprosy patients. Int. J. Lepr. **52** (1984) 183–188.
2. Dhople, A. M. and Hanks, J. H. Quantitative extraction of ATP from cultivable and host grown microbes: Calculations of ATP pools. Appl. Microbiol. **26** (1973) 399–403.
3. Dhople, A. M. and Hanks, J. H. Adenosine triphosphate of *M. leprae.* A brief communication. Int. J. Lepr. **49** (1981) 57–59.
4. Dhople, A. M. and Storrs, E. E. Adenosinetriphosphate content of *M. leprae*: Effect of purification procedures. Int. J. Lepr. **50** (1982) 83–89.
5. Hanks, J. H., Chatterjee, B. R. and Lechat, M. F. A guide to the counting of mycobacteria in clinical and experimental materials. Int. J. Lepr. **32** (1964) 154–167.
6. Heinen, H. J., Hillenkamp, F., Kaufmann, R., Schroeder, W. and Wechsung, R. LAMMA: A new laser microprobe mass analyser for biomedicine and material analysis. In: *Recent Developments in Mass Spectrometry in Biochemistry and Medicine.* Frigerio, A. and McCamish, M., eds. Amsterdam: Elsevier Science Publ., 1980, vol. 6, pp. 435–459.
7. Lindner, B. and Seydel, U. Mass spectrometric analysis of drug-induced changes in Na^+- and K^+-contents of single bacterial cells. J. Gen. Microbiol. **129** (1983) 51–55.
8. Lindner, B. and Seydel, U. Results on taxonomy and physiological state of bacteria derived from laser-induced single cell mass analysis. J. Phys. Colloque (Paris) **45** (C2) (1984) 565–568.

9. SEYDEL, W. and HEINEN, H. J. First results in fingerprinting of single mycobacterial cells with LAMMA. In: *Recent Developments in Mass Spectrometry in Biochemistry and Medicine.* Frigerio, A. and McCamish, M., eds. Amsterdam: Elsevier Science Publ., 1980, vol. 6, pp. 489–496.
10. SEYDEL, U., and LINDNER, B. Qualitative and quantitative investigations on mycobacteria with LAMMA. Fresenius Z. Anal. Chem. **308** (1981) 253–257.
11. SEYDEL, U., LINDNER, B., SEYDEL, J. K. and BRANDENBURT, K. Detection of externally induced impairments in single bacterial cells by laser microprobe mass analysis. Int. J. Lepr. **50** (1982) 90–95.
12. SHEPARD, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot pads of mice. J. Exp. Med. **112** (1960) 445–454.