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Detection of Externally Induced Impairments in Single Bacterial Cells by Laser Microbe Mass Analysis[1]

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Leprosy is an infection caused by bacteria which are non-cultivable *in vitro*. Only a limited amount of bacteria from biopsies is available to monitor for relapses occuring during therapy. This is usually performed via the mouse foot pad test ([9]), a procedure which is rather time consuming and not always confident. It would therefore be desirable to develop a method capable of tracing alterations in single cells induced by external treatment.

A promising way in this direction seems to be offered by a new microanalytical technique, laser microprobe mass analysis (LAMMA). This technique allows the measurement of mass spectra of atomic and molecular ions from single bacterial cells with extremely high sensitivity. Earlier investigations on the classification of different mycobacterial strains by single cell analysis have demonstrated that mycobacteria give rise to a typical pattern for their mass fingerprints differing only in certain mass peaks ([5]). These patterns can be altered by external influences, e.g., temperature. This is especially true for the K^+/Na^+-ratio which is a very sensitive indicator of viability. To verify these initial findings a Gram negative species (*E. coli*) and various mycobacterial strains (*M. tuberculosis* H37Ra, "*M. lufu*," and *M. leprae*) were treated either with high temperature, X-radiation, isonicotinic acid hydrazide (INH), or dapsone (diaminodiphenylsulfone; DDS) and analyzed by the LAMMA-technique as described elsewhere ([7]).

In two cases (X-irradiation and INH-treatment of *M. tuberculosis* H37Ra) the microanalytical findings were supported by determining the number of viable cells via the agar plate culture method. Also, for heat and INH-treated bacteria the Na^+- and K^+-content was measured by atomic absorption spectroscopy (AAS). It will be shown that the application of the LAMMA-technique to analyze single bacterial cells can provide a tool for monitoring therapy in leprosy.

MATERIALS AND METHODS

The following bacterial strains were used: *E. coli* (mutaflor), *M. tuberculosis* H37Ra, "*M. lufu*" L-209, and *M. leprae*. "*M. lufu*" has been isolated by Portaels ([4]) and is not yet completely classified. It was utilized because of its similar sensitivity pattern to certain inhibitors of *M. leprae*. Also the growth rate of "*M. lufu*" is very slow and its temperature for maximum growth is similar to that of *M. leprae* (31°C).

GROWTH

***E. coli* mutaflor.** The procedure described by Seydel, *et al.* ([8]) was followed: a broth culture was inoculated from agar culture in Anton-medium ([1]) and allowed to grow for 16 hr at 37°C. A dilution of this preliminary culture was allowed to grow into the exponential phase. At a concentration of about 10^7–10^8 cells/ml (measured by the Coulter Counter technique) replicate volumes of broth were inoculated in Erlenmeyer flasks.

***M. tuberculosis* H37Ra.** Stock cultures were maintained on Löwenstein-Jensen slants at 37°C. Dubos media (1000 ml) with-

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out albumin in penicillin flasks were inoculated with heavy cell suspensions (50 g) of 7 days old cultures. After incubation the final cultures were allowed to grow for 14 days at 37°C.

"M. lufu." A broth culture was inoculated from 4–6 week old agar cultures on Gottsacker in a modified Dubos medium (containing 0.05% Tween 80, 0.25% fraction V bovine serum albumin) with a starting concentration of 10^5 cells/ml and grown at 31°C up to a density of approximately 10^7 cells/ml. The bacteria were harvested, homogenized in medium (5 ml), the suspension diluted with medium (15 ml), and centrifuged (150 × *g* × 4 min). Part of the supernatant was taken for electronic counting and the final culture (50 ml in 300 ml Erlenmeyer flasks) inoculated with approximately 10^5 cells/ml. During growth the cultures were stirred magnetically.

M. leprae. The bacteria were a generous gift from the IMMLEP Programme of WHO. They were isolated from tissues (lymph nodes, dissected from fat) of infected armadillos and sterilized by a 2.5 Mrd dose of ^{60}Co-radiation.

Treatment. *E. coli* cells were treated with dapsone (diaminodiphenylsulfone, DDS); *M. tuberculosis* H37Ra with isonicotinic acid hydrazide (INH), X-rays, and heat; "*M. lufu*" with DDS; *M. leprae* were inactivated by ^{60}Co-irradiation. DDS and INH (Bayer AG, Leverkusen) have been chosen because of the high effectiveness of DDS against *M. leprae* and "*M. lufu*" and of INH against *M. tuberculosis*.

DDS was applied to the final cultures (*E. coli*, "*M. lufu*") after inoculation in a concentration of 300 μmol/l. The same procedure was applied for the INH-treatment of H37Ra, but at a concentration of 10 μmol/l. All cultures were then allowed to grow up to the stationary phase.

One additional culture of H37Ra in the stationary phase was heated up to 73°C in about 2.5 hr, and another was irradiated with 300 keV X-rays at a dose rate of 0.72 Gy* min^{-1} for about 2 hr to yield a final dose of 83.8 Gy.

In each of the experiments an untreated culture was kept as a control under identical conditions except for treatment. From all cultures, treated and untreated, the bacteria were harvested by sedimentation at 2000 × *g* for 10 min. The bacteria were washed four times in double distilled water at 20°C.

For LAMMA-analysis one drop of bacterial suspension (wet bacterial sediment resuspended in water and stirred thoroughly) was brought onto formvar-filmed Cu-grids (as used for electron microscopy) and the fluid was sucked up by fleece. In this way a widespread distribution of the bacterial cells was achieved allowing the laser vaporization of one single cell at a time (see below). This procedure was slightly different for *M. leprae*. Here small amounts of the freeze-dried bacteria were ground between two cover glasses and then spread on the filmed side of the Cu-grids.

Viable cell count. Samples of the cultures (irradiated and INH-treated H37Ra) were diluted with Dubos medium to give final concentrations between 10^{-4} and 10^{-8} (control) or 10^{-3} and 10^{-7} (treated culture), respectively. For each concentration five tubes with Löwenstein-Jensen nutrient were inoculated. The tubes were incubated at 37°C for 5 weeks and the resulting colonies counted.

Atomic absorption spectroscopy. The wet sediment of controls and of heat- and INH-treated H37Ra cultures was dried at 105°C to constant weight and digested in 100 μl of concentrated sulfuric acid and 200 μl of hot nitric acid. For quantification the absorption values of the dissolved bacterial samples were compared with those of standard Na^+- and K^+-solutions containing small amounts of CsCl to suppress quenching.

Laser Microprobe Mass Analyser (LAMMA). The LAMMA instrument has been described in detail elsewhere ([2]). Briefly, the instrument represents a combination of a laser microscope with a time-of-flight (TOF) mass spectrometer. The microscope serves both for observation of the specimen to be investigated and for the focusing of an UV-pulse laser beam (λ = 265 nm) onto the specimen (spot size ≈ 1 μm in diameter). The interaction of the laser beam with the specimen leads to the formation of positive and negative atomic and molecular

* Gy = Gray (International system standard unit; measure of the amount of radiation absorbed by matter; equivalent to 1 joule of energy).

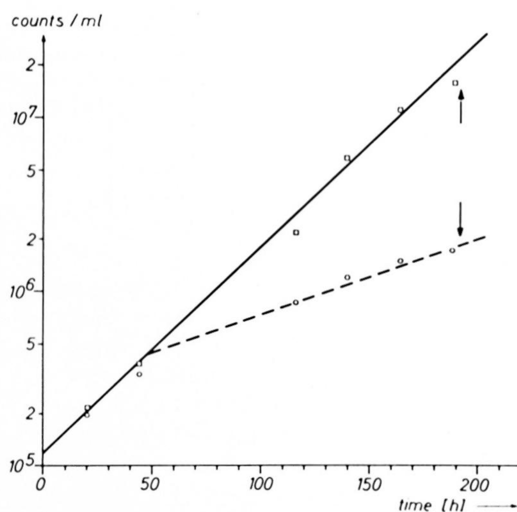

FIG. 1. Example of the growth kinetics of "*M. lufu*" followed by the Coulter Counter technique in the absence (□——□) and presence (○----○) of dapsone (diaminodiphenylsulfone, DDS). The time point of harvesting bacteria for microprobe mass analysis is marked by arrows. The drug was added at t = 0.

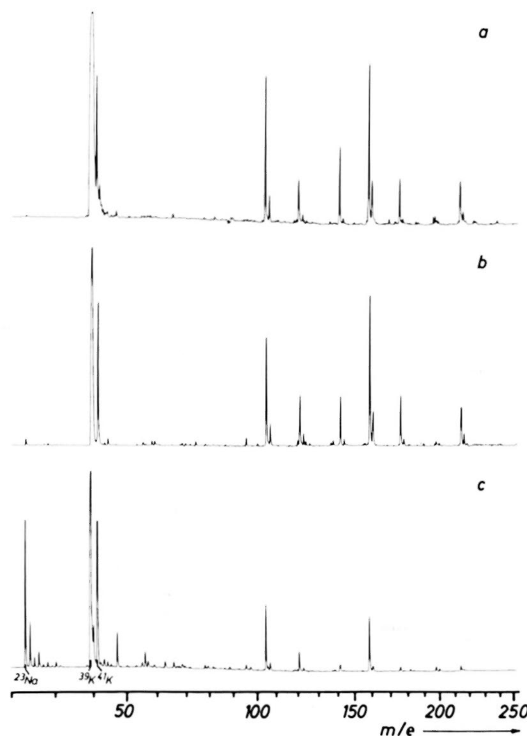

FIG. 2. LAMMA-spectra of single *M. tuberculosis* H37Ra cells not treated (a) or treated by 10 krd X-irradiation (b, c).

ions which are detected alternatively by the TOF-mass analyser (mass resolution up to 1000). The detection limit reaches 10^{-20} g for some elements (Na, K) in an analyzed sample volume of 3×10^{-13} cm^3 for organic matrix material prepared as thin sections (thickness 0.1–1 μm) ([2]).

RESULTS AND DISCUSSION

Figure 1 shows as an example the growth kinetics of "*M. lufu*" followed by Coulter Counter technique in the absence and presence of inhibitor (DDS). The time point of harvesting the bacteria for microprobe mass analysis is indicated. From each preparation, control or treated samples, about 20 single cell mass spectra were obtained. In Figures 2–6 examples are given for the different bacteria used and treatment applied.

Major peaks were seen at m/e = 39, 41, and in some cases 23, corresponding to $^{39}K^+$, $^{41}K^+$ and $^{23}Na^+$, respectively. In many of the spectra of damaged cells, a peak occurs at m/e = 40, which most probably has to be attributed to $^{40}Ca^+$. In the spectra taken from mycobacteria (Figs. 2, 3, 4, 6) the peaks at m/e >100 have a pattern which has already been found to be typical for other mycobacterial strains ([5]). The differences in this mass spectral region with mycobacteria compared to the pattern of *E. coli* are obvious from Figure 5a.

For the sake of clearness, only typical patterns for the cases of extreme or small alterations are shown in the figures. Between these extremes, in most cases, a great variety of minor deviations from the patterns of untreated cells could be found.

Figure 2 shows the results obtained with H37Ra cells which had been irradiated by a 10 krd dose of X-rays. The fingerprint of an untreated cell (Fig. 2a) is compared with two fingerprints of irradiated cells (Fig. 2b, c). In one case (b) no major deviations from the control can be seen, whereas from (c) it becomes obvious that the K^+/Na^+-ratio is shifted to a smaller value and that the intensity of mass peaks for m/e-values higher than about 100 is considerably lower as compared to (a). The fact that two different types of patterns are produced by X-irradiation could be explained by target

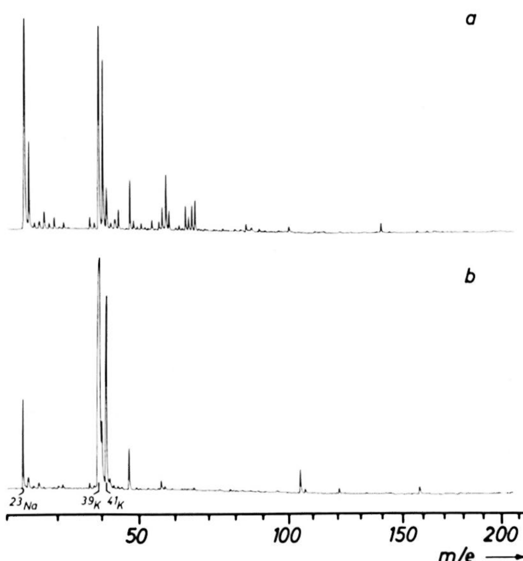

FIG. 3. LAMMA-spectra of single *M. tuberculosis* H37Ra cells treated by heat (a) or by isonicotinic acid hydrazide (b).

theory, according to which the damage to a single cell depends on probability factors, i.e., one cell may be damaged whereas another one is left totally unaffected ([3]). This is also supported by the results from the viable cell count method, according to which about 40% of the cells survived the 10 krd-X-irradiation.

Figure 3a shows a typical fingerprint of a heat-treated and Figure 3b that of an INH-treated *M. tuberculosis* H37Ra cell. Both patterns are similar and resemble that of Figure 2c (irradiated cells). Again an increase in the Na^+-intensity, a decrease of the K^+-intensity (^{41}K) and a suppression of higher mass peaks is seen.

The AAS-measurements, in fact, support the findings from LAMMA-application. For the heat-treated bacteria, a pronounced decrease in the K^+/Na^+-ratio was observed, whereas, in contradiction to the results of Urbanczik ([10]), the INH-treated bacteria showed only a minor shift in this ratio. In Figure 4 spectra of "*M. lufu*" treated with DDS (Fig. 4b, c) as compared to non-treated controls (a) are shown. Again, two different patterns are obtained for treated cells. The spectrum 4b resembles that of the control (the lower intensities of all mass peaks might be explained by a lower excitation by the laser beam due to an insufficient focusing). The spectrum 4c again shows the typical patterns of damaged cells with the intense Na^+-peak. Here the occurrence of two different patterns after treatment (4b, c) is, for the present, surprising. It might be due to clumping of "*M. lufu*" in culture, inhibiting the drug from penetrating into some of the cells. During preparation for LAMMA-analysis, these clumps are partially disrupted, so that in some cases untreated cells could have been hit by the laser beam.

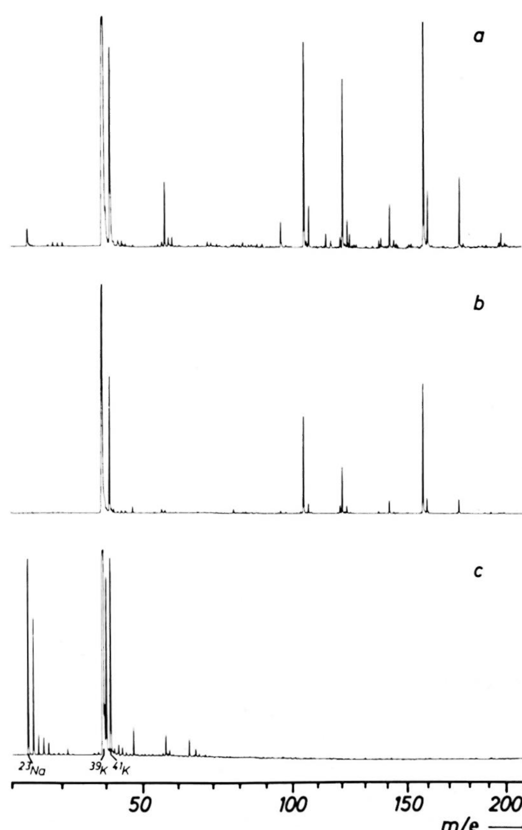

FIG. 4. LAMMA-spectra of single "*M. lufu*" cells not treated (a), or treated by dapsone (diaminodiphenylsulfone) (b, c).

Spectra obtained with *E. coli* controls and cells treated with DDS, are presented in Figure 5. As stated before, the mass spectrum of an untreated cell (Fig. 5a) shows a different pattern from that of a mycobacterial cell, especially for m/e-values higher than about 100. The influence of

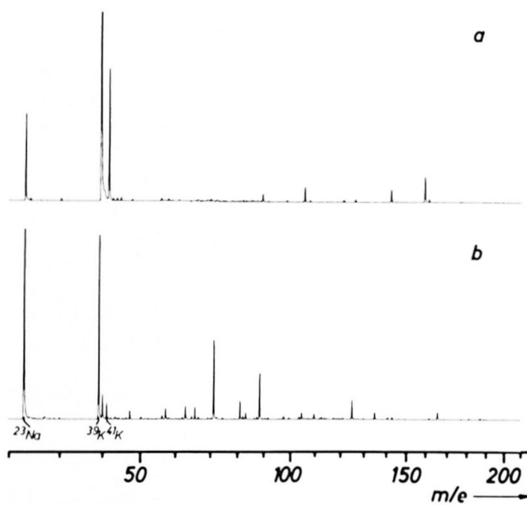

FIG. 5. LAMMA-spectra of single *E. coli* cells not treated (a), or treated by dapsone (diaminodiphenylsulfone) (b).

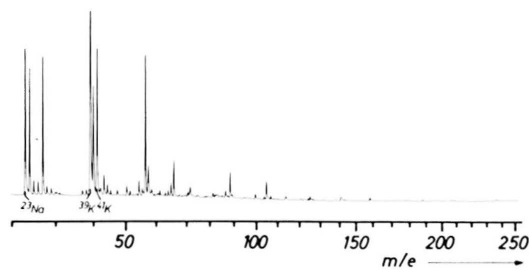

FIG. 6. LAMMA-spectrum of a single *M. leprae* cell isolated from tissue and sterilized by 2.5 Mrd ^{60}Co-irradiation.

the drug again manifests itself in an increase in the Na^+-intensity and in a very pronounced decrease in the K^+-intensity, however.

Figure 6 gives a first example of a mass spectrum of a single *M. leprae* cell isolated from armadillo tissue. The pattern is similar to those of the other damaged cells (Figs. 2c, 3a, b, 4c) in that it contains a large peak corresponding to Na^+ and in that peaks in the high m/e-region are of low intensity. However, the peaks at the m/e-values "typical" for mycobacteria cannot be recognized conclusively. It cannot be determined at present, however, whether the damage occurred during isolation from tissue or during sterilization by ^{60}Co-irradiation.

If the mass spectra (fingerprints) of untreated mycobacterial cells presented in this paper are compared with those published earlier ([5]), the large scale reproducibility of the method becomes obvious. However, the preliminary nature of our present studies has to be emphasized. The fact that a computer was not available for spectral analysis put some restrictions on the number of experiments which could be performed. Nevertheless, the results indicate that it is possible to distinguish between viable and damaged cells by fingerprinting single cells. It should be kept in mind that a chemotherapeutic treatment of a mycobacterial infection like leprosy will certainly lead to a wide gradation in the alterations detectable in the mass spectra. Studies on the influence of drugs on bacteria must be based on the evaluation of large numbers of single cell spectra in order to comprehend distribution patterns of distinct atomic or molecular peaks and to compare the distribution patterns under investigation with the respective patterns of the control.

The mass spectra shown here consist only of a very limited number of mass peaks and are therefore quite simple. This is because a very low laser power density was applied. An increase in laser power density would lead to much more complex spectra ([2]), the analysis of which would require computer analysis.

In spite of these drawbacks it seems likely that the laser microprobe mass analysis of single mycobacterial cells can be a promising tool for monitoring responses to therapy in infections where *in vitro* growth of the infecting organisms is not possible.

In a next and more difficult step, an attempt should be made to attribute certain mass peaks to the appropriate molecules or molecular fragments. This attempt might yield information on the mechanism of action and metabolism of a certain chemotherapeutic drug.

SUMMARY

Applying the laser microbe mass analyzer method (LAMMA), mass spectra (fingerprints) were taken from single bacterial cells not treated or treated with high temperature, X-irradiation, isonicotinic acid hydrazide (INH), or diaminodiphenylsulfone (DDS). Spectra of treated cells ("*M. lufu*," *M. tuberculosis* H37Ra, *E. coli*) dif-

fer from those of controls in that the K^+/Na^+ ratio was smaller and in that the intensities of peaks with m/e > 100 were lower. From the results with *M. leprae* the possible application of this new method for monitoring the effectiveness of leprosy therapy is proposed.

RESUMEN

Usando el analizador laser de masa microbiana (LAMMA), se tomaron "las huellas digitales" (espectros de masa) de células bacterianas aisladas no tratadas, o tratadas con: temperaturas elevadas, irradiación-X, la hidrazida del ácido isonicotínico (INH), o con diaminodifenil sulfona (DDS). Los espectros de las células tratadas (*M. lufu, M. tuberculosis* H37Ra y *E. coli*) difirieron de aquellos de los controles no tratados en que en los primeros, la relación K^+/Na^+ fue menor y los perfiles de los picos con relaciones m/e > 100 fueron diferentes. Por los resultados obtenidos con el *M. leprae*, se propone la posible aplicación de este nuevo método para seguir la efectividad de la terapia antileprosa.

RÉSUMÉ

Au moyen d'une méthode d'analyse de la masse microbienne au laser (LAMMA), les spectres de masse (empreintes) ont été établis pour des cellules bactériennes isolées non traitées, ou traitées par diverses méthodes: température élevée, irradiation par Rayon-X, hydrazide de l'acide isonicotinique (INH) et diaminodiphenylsulfone (DDS). Les spectres des cellules traitées (*M. lufu, M. tuberculosis* H37Ra, *E. coli*) étaient différents de ceux fournis par les témoins à deux égards: le rapport K^+/Na^+ était plus faible et les dépressions des valeurs maximales avec m/e > 100 étaient moins élevées. Sur la base des résultats obtenus avec *M. leprae*, on propose une application possible de cette nouvelle méthode pour suivre l'efficacité de la thérapeutique antilépreuse.

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