INTERNATIONAL JOURNAL OF LEPROSY

Volume 69, Number 1
Printed in the U.S.A.
(ISSN 0148-916X)

Limited ATP Generation in Cells of *Mycobacterium leprae* Thai-53 Strain in Enriched Kirchner Liquid Medium Containing Adenosine[1]

Masahiro Nakamura and Masanori Matsuoka[2]

No credible or reproducible *in vitro* culture method has yet been reported for *Mycobacterium leprae* obtained from leprosy patients or derived from experimentally infected mouse foot pads, either in a tissue culture or in a cell-free culture system, even though it has now been more than 120 years since the discovery of the bacilli as the causative agent of leprosy by Hansen in 1873.

For cultivation experiments, three parameters, i.e., the number of bacilli, the intracellular adenosine triphosphate (ATP) content, and the DNA content extracted from cultured cells, are essential for evaluating the multiplication of the bacilli.

Evidence is presented in this paper indicating that ATP generation can be produced in cells of *M. leprae* derived from experimentally infected mouse foot pads when the bacilli are inoculated into enriched Kirchner liquid medium containing adenosine and cultured at 30°C . Factors affecting the ATP generation in *M. leprae* cells and the related problems concerning this discovery are reported and discussed.

MATERIALS AND METHODS

M. leprae suspension

The Thai-53 strain ([8]), propagated and serially maintained in nude mice (BALB/c-*nu/nu*) foot pads, was used throughout. An *M. leprae* suspension was prepared as previously described ([10]). Briefly, foot pads of *nu/nu* mice experimentally infected at 11–13 months previously with 1×10^6 *M. leprae* Thai 53 strain were surface decontaminated with iodine tincture for 10–15 sec and then rinsed in 70% ethanol. The foot pads were then minced with scissors and homogenized using a glass homogenizer in sterile 0.05 M phosphate buffer (pH 7.0). After the coarse tissue debris was removed by low-speed centrifugation ($10 \times g \times 20$ sec), sterile 0.5% w/v trypsin solution (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was added to the bacillary suspension at a final concentration of 0.05%. The mixture was incubated in a water bath at 37°C for 60 min and then centrifuged ($1500 \times g \times 15$ min) to remove the trypsin. The sediment was resuspended in 0.05 M buffer (pH 7.0) and treated with NaOH at a final concentration of 0.25 N for 15 min in a water bath at 37°C. After this treatment, the mixture was centrifuged at $1500 \times g \times 7$ min. The final bacterial suspension was obtained by resuspending the pellet in 0.05 M phosphate buffer (pH 7.0) containing fetal calf serum (20% v/v).

The suspension thus prepared contained mostly bacilli but also an extremely small amount of tissue contaminants. No reduction in the activity compared to that of the starting material was found by inoculation into mouse foot pads. The intracellular ATP contents of *M. leprae* employed in the experiments ranged from 2.4×10^{-16} g to 2.9×10^{-17} g/bacillus.

Culture medium

The culture medium (NK-260) was designed on the basis of the composition of Kirchner medium ([6]) and adopted from the results obtained in studies on cultivation of *M. lepraemurium* ([9]) and on the preservation of the activity of *M. leprae* under cell-

[1] Received for publication on 5 July 2000. Accepted for publication in revised form on 13 February 2001.

[2] M. Nakamura, M.D., Ph.D., Professor Emeritus, Kurume University, Koga Hospital Medical Research Institute, 120 Tenjin, Kurume 830-8577, Japan. M. Matsuoka, Ph.D., National Institute of Infectious Diseases, Leprosy Research Center, 4 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan.

Reprint requests to Dr. Nakamura at the above address or FAX 81-942-38-2255; e-mail: akikokhp@kurume.ktarn.or.jp

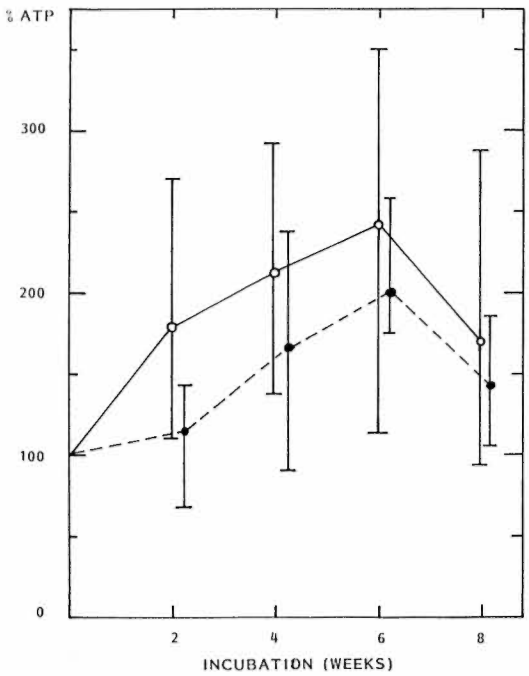

FIG. 1. ATP generation in *M. leprae* cells in the enriched Kirchner medium with adenosine (at 50 μg/ml) (○) and without adenosine (●) cultured at 30°C (N = 14).

free conditions ([11]). The composition of the basal medium was as follows: KH_2PO_4 0.3%, Na_2HPO_4 0.6%, sodium citrate 0.25%, $MgSO_4$ 0.06%, glutamate 0.8%, glycerin (92.09%, Wako Co., Japan) 2%, and adenosine 50 μg/ml. The pH was adjusted to 7.0 with 5 N KOH, and the medium was autoclaved. The following supplements were aseptically added to the sterile basal medium: 10% fetal calf serum (GIBCO BRL, Gaithersburg, Maryland, U.S.A.), 10% egg-yolk extract (v/v), and pyruvate and transferrin at a final concentration of 0.2% and 10 μg/ml of the medium, respectively. The 10% egg-yolk extract was prepared by adding one volume of egg yolk (purchased at a grocery) to 9 volumes of water, mixing, and then keeping the extract in a refrigerator overnight. The supernatant was sterilized by filtration, first with a 0.4-μm and then with a 0.2-μm Millipore filter.

Inoculation of *M. leprae* cells and incubation conditions

One milliliter of a suspension containing 3.3–5.6 × 10^8 *M. leprae* was inoculated into 60 ml of the culture medium, and 5 ml aliquots were then put into test tubes (90 mm × 12 mm screw-cap tubes) leaving a 25% free air space above the medium, and then cultured at 30°C.

Evaluation of growth

Growth of *M. leprae* was evaluated by the three parameters: 1) bacillary counting, 2) the ATP content extracted from the cultivated cells, and 3) the DNA content of cells amplified by the PCR.

Bacillary counting. Bacillary counting was carried out by the method of Shepard and McRae ([13]).

ATP extraction. To measure the intracellular ATP content of the cultured cells, the cells were collected by centrifugation, and the ATP then was extracted from the pellet. ATP determinations were carried out by the firefly bioluminescent technique previously described ([2]).

Polymerase chain reaction (PCR). The bacilli collected by centrifugation were resuspended in 1 ml of phosphate buffered saline (PBS) (0.01 M, pH 7.2) containing Tween 80 (0.05%) for loosening natural aggregation and clumping. The suspended samples from all incubation periods were stored at –20°C until the termination of each experiment at which time PCR was carried out on all samples at the same time. Samples were serially diluted twofold, and 50 μl of each dilution was used for templates. The serially diluted samples were frozen at –30°C and thawed in boiling water. This procedure was repeated three times. A portion of the *M. leprae*-specific repetitive element was amplified according to the methods of Wang and Mark ([14]) and Woods and Cole ([16]). In our experiments, however, the annealing temperature used was 60°C, and an amplification of 20 times of the thermal cycle was employed. Samples of the PCR products (8 μl) were subjected to electrophoresis in 2% agarose gels followed by visualization with ethidium-bromide staining and ultraviolet irradiation.

Bacteriological examinations

The *M. leprae* used in all inocula, as well as the *M. leprae* from all *in vitro* cultures harvested and contamination checks, were confirmed on the basis of the following criteria: acid-fastness by Ziehl-Neelsen stain-

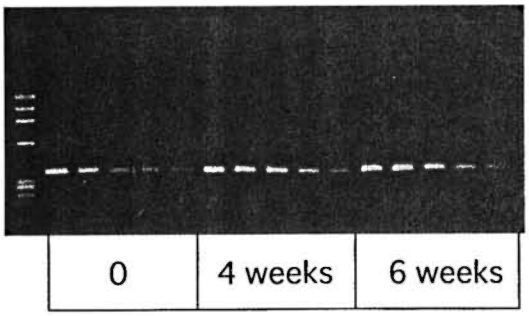

FIG. 2. PCR products from cells of *M. leprae* cultured in the enriched Kirchner medium containing adenosine (50 μg/ml) at 30°C. Bands represent, from left to right, the size markers, undiluted, and twofold diluted samples cultured for 0 weeks, 4 weeks and 6 weeks.

ing, inability to grow on blood agar or on Ogawa egg-yolk slants, pyridine extraction of acid-fastness of stained bacilli and the PCR method.

Renewal of culture medium

Culture tubes cultivated for 6 weeks were centrifuged at 1500 × *g* × 20 min. The supernatants were completely discarded. Collected sediments were then resuspended in freshly prepared culture medium of the same composition as the original starting one (G-1) at a volume equal to the volume of the culture medium centrifuged. The culture material thus prepared (G-2) was distributed at 5 ml per tube and recultivated at 30°C. The ATP contents were measured by the routine method.

RESULTS

ATP generation

A significant increase in the intracellular ATP content of cells of *M. leprae* took place when the cells were inoculated into the enriched Kirchner medium containing adenosine (50 μg/ml) and incubated at 30°C, as shown in Figure 1. The increased rates were more than two- to threefold compared to that of the starting samples, and the increase reached a maximum 4 or 6 weeks after cultivation. These findings were also supported by an increase in the DNA content extracted from cultured cells and amplified by PCR, as shown in Figure 2. On the other hand, an increase in the intracellular ATP content of cells in the enriched

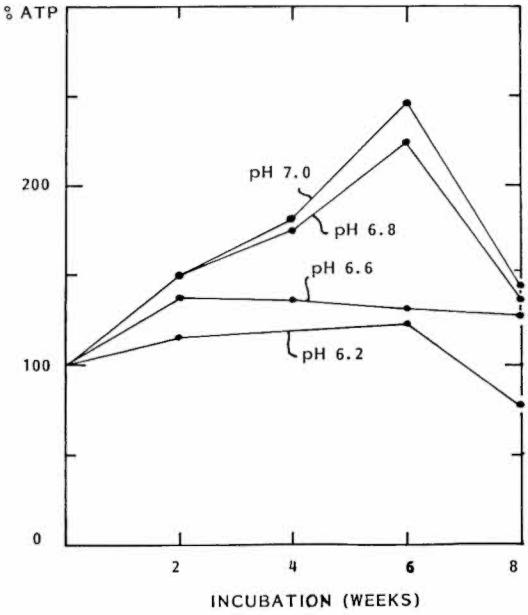

FIG. 3. Effect of pH of the enriched Kirchner medium containing adenosine (50 μg/ml) on ATP generation in *M. leprae* cells.

Kirchner medium without adenosine was less than twofold compared to the starting samples. In general, there were large variations in the ATP content depending upon the quality and quantity of the inoculum (N = 14), e.g., age of bacilli until harvest from inoculation to mouse foot pads and the inoculum sizes. Statistical analysis between the ATP content in cells cultivated in the enriched Kirchner medium with and without adenosine was performed by the Mann-Whitney U test. The results demonstrated that there was a statistical significance indicating $p < 0.0001$ and an F value of 7.667 at 2 and 4 weeks' cultivation, but no statistical significance at 6 and 8 weeks' cultivation.

Optimal concentration of adenosine

The results obtained in repeated experiments indicated that the concentration of adenosine for the ATP generation was optimal at 50 μg/ml of the culture medium and that at 15 μg it was not effective, at 75 μg was less effective, and at more than 100 μg the concentration of adenosine was toxic.

Bacillary counting

No significant increase in the number of bacilli in the enriched Kirchner medium containing adenosine (50 μg/ml) was observed.

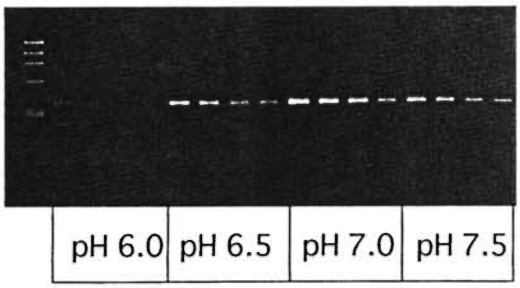

FIG. 4. Effect of pH of the medium on PCR products. Bands represent, from left to right, the size markers, undiluted, and twofold diluted DNAs extracted from cells cultured in the medium of pH 6.0, 6.5, 7.0 and 7.5, for 6 weeks at 30°C.

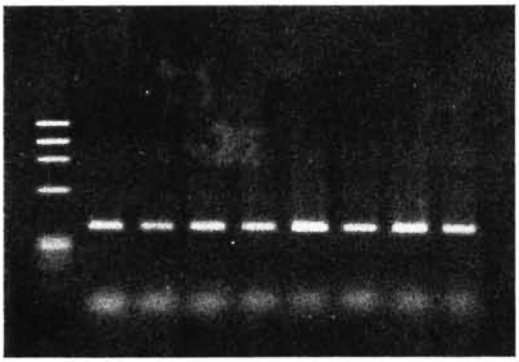

FIG. 5. PCR products from cells of *M. leprae* cultured in enriched Kirchner medium (pH 7.0) without adenosine and the medium (pH 6.6) with adenosine. Bands represent, from left to right, the size markers (1), undiluted and twofold diluted DNAs extracted from cells incubated in the enriched Kirchner medium without adenosine (2–5), and the medium (pH 6.6) with adenosine (6–9) for 6 weeks at 4°C and 30°C, respectively.

Factors affecting ATP generation

pH of medium. The pH of the medium is one of the important factors for the ATP generation in cells of *M. leprae*. The typical experimental results demonstrated, as shown in Figure 3, that the most effective pH was 7.0 for *M. leprae* and that a pH of 6.2 was not effective—a pH of 6.2 is the critically effective pH for the growth of *M. lepraemurium*. The effect of pH was also demonstrated to be identical in the results evaluated by PCR, as shown in Figure 4.

Other factors tested. No ATP generation in the cells of *M. leprae* cultured in the enriched Kirchner medium (pH 7.0) without adenosine and in the medium (pH 6.6) with adenosine was observed (Fig. 5). Furthermore, no effect of adenosine on the ATP generation was demonstrated in a phosphate buffer solution (pH 7.0) containing 10% FCS and the original Kirchner liquid medium. As shown in Figure 6, no increase in the DNA content of incubated cells estimated by PCR was observed in the original Kirchner liquid medium (at pH 6.6 or at pH 7.0) incubated at 4°C or at 30°C for 6 weeks. No ATP generation in the enriched Kirchner medium containing adenosine occurred when an inoculum containing less than 10 ng ATP/tube was used, or when the medium was incubated at 37°C. Antileprosy drugs, such as dapsone (DDS), streptomycin, or rifampin each (6 μg/ml), inhibited the ATP generation. Adenosine monophosphate and adenosine 5′-diphosphate are able to replace adenosine, but adenine was not effective. As far as ATP was concerned, there were practical difficulties in the removal of adhered ATP from the cell wall of the bacillus.

Effect of renewal of medium on ATP generation. In general, the results obtained so far consistently indicate that ATP generation reached a maximum 4 or 6 weeks after cultivation, and the amount of ATP was

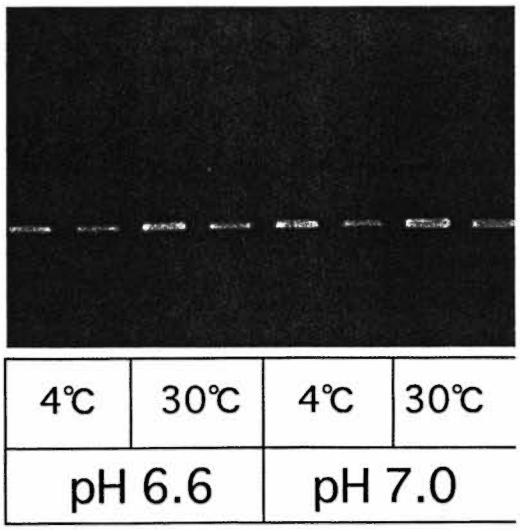

FIG. 6. PCR products from cells of *M. leprae* cultured in the original Kirchner medium of pH 6.6 and 7.0. Bands represent, from left to right, fourfold and eightfold diluted DNAs extracted from cells incubated in the medium for 6 weeks at 4°C and at 30°C.

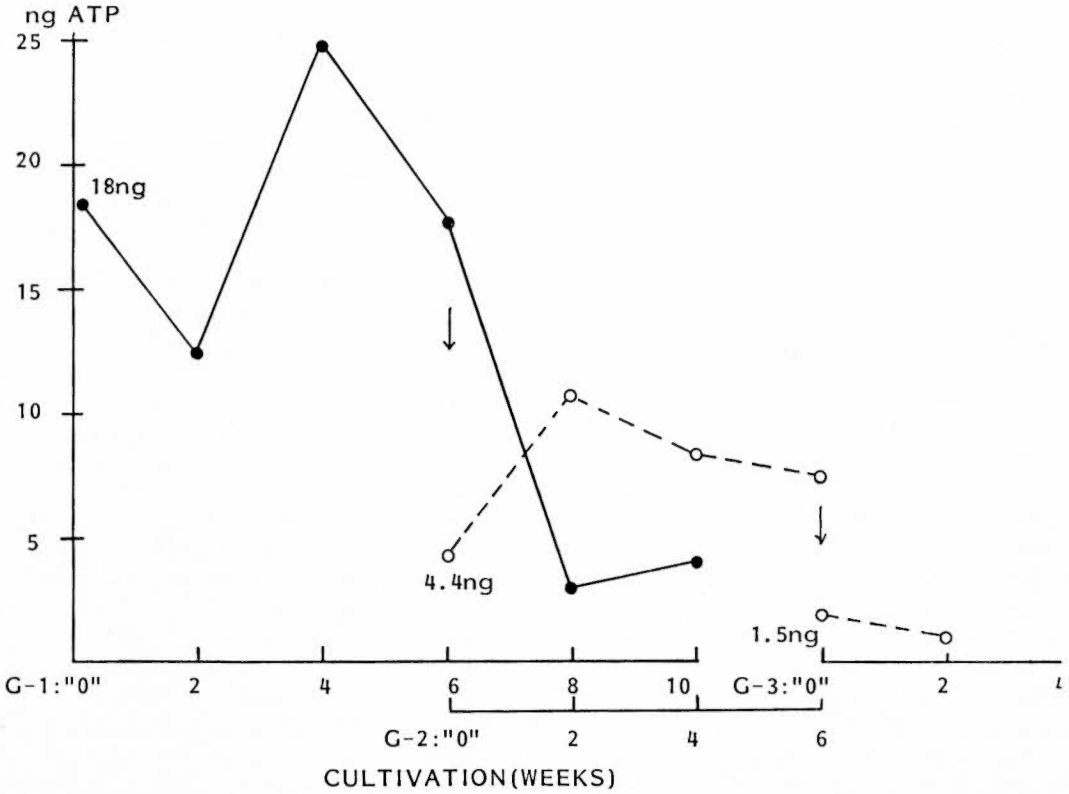

FIG. 7. Effect of renewal of the culture medium on the ATP gerneration in cells of *M. leprae*.

reduced thereafter. Therefore, to further maintain and progressively increase the ATP generation, a renewal of the cultured medium using freshly prepared culture medium was tried at 6 weeks after cultivation. The results of the renewal trial of the culture medium are shown in Figure 7. The ATP content in cells per tube represents ng/tube. It is demonstrated that the ATP content in cells cultivated for 6 weeks markedly decreased just by transferring the cells from the primary culture medium (G-1) to the freshly prepared culture medium (G-2): a 74% loss in ATP content in G-2 compared to G-1. Furthermore, a 79% loss in ATP content by transfer from G-2 to G-3 was observed. In addition, morphological observations demonstrated that the number of nonsolid forms in the cells increased compared to that of the cells primary culture (G-1) for 6 weeks.

DISCUSSION

For evaluation of the growth of *M. leprae*, a bacillary counting method is essential. The growth of *M. leprae* in mouse foot pads or in armadillos has been routinely estimated by the bacillary counting method. However, this bacillary counting method is not suitable in *in vitro* cultivation experiments because mycobacterial cells naturally aggregate and are almost impossible to disperse. Moreover, this bacillary counting method is not suitable when an extremely slight multiplication of bacilli takes place.

Because a close and parallel relationship exists between the increase in the number of bacilli and the increase in the amount of ATP extracted from the cultivated bacilli, it is recognized that an increase in the intracellular ATP content during cultivation quantitatively indicates the multiplication of the cultivated bacilli. In the case of *M. lepraemurium*, ATP measurement of cultured cells has been routinely employed for an estimation of growth and for researching any subtle effect of an ingredient on the growth of the bacilli; this procedure is more suitable than the bacillary counting method for this purpose ([3]). Our results indicate that

the intracellular ATP content in the cells of *M. leprae* consistently increased in the medium containing adenosine after 4–6 weeks of cultivation, and decreased thereafter. Results similar to our findings were previously reported in a study by Katoch, *et al.* ([4]), in which there was a marginal increase in the intracellular ATP in *M. leprae* cells initially in a medium containing asparagine or glycerol, but their increase was not sustained and there was no progressive multiplication. In their studies, the effect of adenosine was not tested.

Our results strongly suggest that the cells of *M. leprae* multiplied approximately more than two times but less than four times in the enriched Kirchner medium containing adenosine during 4–6 weeks' cultivation at 30°C. These findings were also supported by the results of the DNA PCR. Among the factors supporting the growth, it was unequivocal that the adenosine played a key role, but other supplements added to the original Kirchner medium might have had some effects on this growth.

This paper has reported an increase in the intracellular ATP of *M. leprae* of more than two times but less than four times. However, the findings are noteworthy because there has been no report to date concerning any reproducible increase in the intracellular ATP content of *M. leprae* derived from experimentally infected mouse foot pads. Dhople ([1]) reported several studies on the multiplication of *M. leprae in vitro*. In those reports, the bacilli were derived from nine-banded armadillos, and no increase in the intracellular ATP content was found when the bacilli were derived from mouse foot pads. In another study, Lee and Colston ([7]) reported that the intracellular ATP content of *M. leprae* isolated from armadillo tissue showed an exponential decrease during cultivation in a nutrient-Tween medium or in a thioglycollate medium and, despite its inability to multiply, *M. leprae* were noted as capable of synthesizing ATP *in vitro*. In addition, Sathish and Nath ([12]), Khanolkar and Wheeler ([5]) and Wheeler ([15]) have reported that purine bases, especially adenosine, were generally incorporated into *M. leprae* at 6 to 20 times the rate of pyrimidines. Hence, evidence presented in this paper might suggest that adenosine could help ATP synthesis of *M. leprae* in some ways, resulting in a reliable increase in the intracellular ATP content of the bacilli during cultivation trials.

The reason why ATP generation ceased 4–6 weeks after cultivation is not clear, but it was determined that the termination in ATP generation was not as a result of deterioration in the culture medium during cultivation because a renewal trial of the old cultured medium by freshly prepared culture medium had no effect on further maintenance or progressive increase in ATP generation. From the results obtained in a renewal trial of the culture medium, we speculate that the reasons why the continuous increase in the ATP content in the cells in this system did not take place, and why *M. leprae* cells do not multiply *in vitro*, might be due to the characteristic properties of the cell wall of *M. leprae*, i.e., fragility, because a remarkable decrease in the ATP content of primarily cultured cells was observed just by a transfer procedure of the cells to a freshly prepared medium, and an increase in the number of nonsolid forms was demonstrated in the transferred cells. As is well known, the role of the bacterial cell wall, in general, protects the cytoplasmic membrane from environmental stress. For such a purpose, the cell wall is extremely rigid. If the cell wall is fragile, stress reaches the cytoplasmic membrane directly, and the cytoplasmic membrane might be destroyed. Consequently, the cessation in energy metabolism and in protein synthesis of the bacterial cell immediately takes place because the cytoplasmic membrane has an important role in bacterial synthesis. Moreover, the cell wall of *M. leprae* has been shown by numerous investigators using electron microscopic observations to be extremely fragile.

SUMMARY

The ATP generation in cells of *Mycobacterium leprae* Thai-53 strain takes place *in vitro* when the cells are cultivated in Kirchner liquid medium, pH 7.0, enriched with egg-yolk solution, pyruvate, transferrin, and adenosine at 30°C. Among the supplements, adenosine was key and critical for the ATP generation. The optimal concentration of adenosine was 50 μg/ml of the medium. ATP generation, however, was

limited; the rates of increase in ATP content extracted from the cells were approximately two- to threefold compared to that of the starting samples, and the increase reached a maximum at 4 or 6 weeks after incubation. No significant ATP generation in *M. leprae* cells was demonstrated in medium at pH 6.2 or pH 6.6, in the original Kirchner medium with or without adenosine, or when cultured at 37°C, or when containing an antileprosy drug. No detectable increase in the number of *M. leprae* cells was observed with the increase in intracellular ATP content and DNA replication. No effect was seen with renewal of the cultured medium by freshly prepared medium at 6 weeks' cultivation on the progressive ATP generation in *M. leprae.*

RESUMEN

La generación de ATP en las células de *Mycobacterium leprae*, cepa Thai-53, ocurre *in vitro* cuando las células se cultivan a 30°C en el medio líquido de Kirchner, pH 7.0, enriquecido con una solución de yema de huevo, piruvato, transferrina y adenosina. Entre los suplementos, la adenosina fue crítica para la generación de ATP. La concentración óptima de adenosina fue 50 µg/ml de medio. Sin embargo, la generación de ATP fue limitada; la tasa de incremento en el contenido de ATP exiraído de las células fue apenas del doble o del triple de la encontrada en las muestras iniciales, y el incremento alcanzó su máximo nivel entre las 4 y 6 semanas de incubación. No se observó una producción significativa de ATP en las células de *M. leprae* cuando éstas se cultivaron (a) en el medio a pH 6.2 ó 6.6, (b) en el medio original de Kirchner, con o sin adenosina, (c) a 37°C, o (d) en el medio conteniendo alguna droga antileprosa. Tampoco se observó ningún incremento en el número de células de *M. leprae* relacionado con el incremento intracelular de ATP o con la replicación del DNA. El cambio del medio de cultivo a las 6 semanas por medio fresco, no tuvo ningún efecto sobre la generación progresiva de ATP en el cultivo *in vitro* de *M. leprae.*

RÉSUMÉ

La production d'adénosine tri-phosphate (ATP) dans les cellules de *Mycobacterium leprae* de souche Thai-53 est obtenue *in vitro* lorsque les cellules sont cultivées dans le milieu de culture liquide de Kirchner à pH 7,0 complété par une solution de blanc d'œuf, de pyruvate, de transferrine et d'adénosine incubé à 30°C. Parmi ces suppléments, l'adénosine était absolument nécessaire pour la production d'ATP. La concentration optimale d'adénosine était de 50 µg/ml dans le milieu de culture. La production d'ATP était cependant limitée en quantité et dans le temps : le taux d'augmentation de la molécule étant d'environ deux à trois fois par rapport à la quantité des énchantillons de départ, cette augmentation atteignant un maximum 4 à 6 semaines après la mise en incubation. Il n'y a pas eu de production significative d'ATP au sein des cellules *M. leprae* lorsqu'elles ont été incubées dans un milieu de culture à un pH de 6,2 ou 6,6; dans un milieu de Kirschner non modifié avec ou sans adénosine; ou lorsque incubé à 37°C; eu encore lorsque le milieu de culture contenait une molécule chimio-thérapeutique active contre la lèpre. Il n'y a pas eu d'augmentation détectable du nombre des cellules *M. leprae* accompagnant l'augmentation de la concentration intracellulaire en ATP et la réplication de l'ADN. Le renouvellement de l'ancien milieu de culture par un milieu de culture fraîchement préparé après 6 semaines de culture n'a pas eu d'effet positif sui la production d'ATP.

Acknowledgment. We are grateful to the late Prof. Emeritus Y. Hagiha for allowing us to use the apparatus for measuring ATP, to Prof. H. Nakayama, Kyushu University, School of Dentistry, for his valuable suggestions, and to Mr. S. Sendo and Mrs. M. Nogita for their technical assistance.

REFERENCES

1. Dhople, A. M. Factors influencing the *in vitro* growth of *M. leprae;* effect of inoculum. Microbios **94** (1998) 103–112.
2. Dhople, A. M. and Hanks, J. H. Quantitative extraction of ATP from cultivable and host grown microbes: calculation of ATP pools. Appl. Microbiol. **26** (1973) 399–403.
3. Dhople, A. M. and Hanks, J. H. Factors that influence the growth of *M. lepraemurium* in the Nakamura system. Int. J. Lepr. **44** (1976) 18–26.
4. Katoch, V. M., Katoch, K., Bharadwaj, V. P., Datta, A. K., Sharma, V. D., Shivannavar, C. T. and Kannan, K. B. Metabolic studies on mycobacteria. V. A preliminary report on the ATP synthesis by mycobacteria including *M. leprae* by using different substrates. Indian J. Lepr. **59** (1987) 163–170.
5. Khanolkar, S. R. and Wheeler, P. R. Purine metabolism in *M. leprae* grown in armadillo liver. FEMS Microbiol. Lett. **20** (1983) 273–278.
6. Kirchner, O. Die Leistungsfahigkeit der Tiefenkultur des Tuberkelbazillus bei Verwenderung besonders geeigneter flussiger Nahrboden. Zentl. Bakteriol. Parasit. Infek. Hyg. **124** (1932) 403–412.
7. Lee, Y. N. and Colston, M. J. Measurement of ATP generation and decay in *M. leprae in vitro.* J. Gen. Microbiol. **131** (1985) 3331–3337.
8. Matsuoka, M., Kohsaka, K. and Dayanghirang, J. A. Characterization of *Mycobacterium leprae* Thai-53 strain. (Abstract) Int. J. Lepr. **60** (1992) 722.
9. Nakamura, M. Multiplication of *Mycobacterium lepraemurium* in cell-free medium contain-

ing *a*-ketoglutaric acid and cytochrome c. J. Gen. Microbiol. **73** (1972) 193–195.

10. NAKAMURA, M. Elimination of contaminants in a homogenate of nude-mouse footpads experimentally infected with *Mycobacterium leprae*. Jpn. J. Lepr. **64** (1995) 47–50.
11. NAKAMURA, M. Optimal pH for preserving the activity of *Mycobacterium leprae* during incubation of cells in a cell-free liquid medium. Int. J. Lepr. **63** (1995) 28–34.
12. SATHISH, M. and NATH, I. The uptake of ^{3}H-thymidine in *M. leprae* inoculated mouse macrophage cultures as a rapid indicator of bacillary viability; factors influencing specificity of the *in vitro* assay. Int. J. Lepr. **49** (1981) 187–193.
13. SHEPARD, C. C. and MCRAE, D. H. A method for counting acid-fast bacteria. Int. J. Lepr. **36** (1968) 78–82.
14. WANG, A. M. and MARK, D. F. Quantitative PCR. In: *PCR Protocols: A Guide to Methods and Applications*. Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J., eds. San Diego: Academic Press, Inc., 1990, pp. 70–75.
15. WHEELER, P. R. Metabolism in *Mycobacterium leprae:* its relation to other research on *M. leprae* and to aspects of metabolism in other mycobacteria and intracellular parasites. Int. J. Lepr. **52** (1984) 208–230.
16. WOODS, S. A. and COLE, S. T. A rapid method for the detection of potentially viable *Mycobacterium leprae* in human biopsies: a novel application of PCR. FEMS Microbiol. Lett. **65** (1980) 305–310.