

Adenosine Triphosphate Content of *Mycobacterium leprae* from Leprosy Patients¹

Arvind M. Dhople²

For 110 years (1873–1983) inability to cultivate *Mycobacterium leprae* *in vitro* has been a major bottleneck in leprosy research, especially in obtaining useful information on the state of bacterial load in patients during chemotherapy. The concept of “persistor”-viable *M. leprae* in treated leprosy patients (²⁶) has added a new dimension to leprosy treatment and control. The occurrence of drug resistance, one of the anticipated outcomes of chemotherapy, has now become well established in lepromatous leprosy treated with dapsone (DDS) since the first report of its kind by Pettit and co-workers (¹⁹). This is of particular importance, since it is quite clear that transmission of resistant bacilli takes place leading to primary dapsone-resistant disease in contacts (^{17, 18}). Therefore, in any leprosy control program the determination of the patient's response to a given therapy and the detection and quantitation of viable as well as drug-resistant bacilli should be given the highest priority.

The only acceptable method presently available to determine the viability and growth of *M. leprae* is its transmission to normal or immunosuppressed mice or rats (^{9, 12, 21, 23}). However, it is most unlikely to be a standard method because a) it is an expensive and somewhat difficult technique for endemic areas, and b) the time interval for obtaining any valid information is extremely long. Therefore, we have undertaken studies employing adenosine triphosphate (ATP) as a biochemical indicator in chemotherapeutic studies on leprosy patients in order to obtain fast and reliable information on the status of *M. leprae* harvested from these subjects. The reasons for adopting ATP as an indicator of viability of *M. leprae* were discussed earlier (^{7, 8}). The

results of the ATP assay were then compared with those in simultaneous mouse foot pad inoculation studies obtained 8–12 months later in order to determine the viability of the organisms.

MATERIALS AND METHODS

Patients. A total of 24 patients were selected randomly from Argentina, Brazil, Surinam, and Thailand. Some of these patients were untreated and others had been on dapsone (DDS) therapy (regular or irregular) for several years. A biopsy specimen (± 5 mm) from each patient was taken and stored at -76°C ; all specimens were shipped to this institute frozen on dry ice.

Suspension of *M. leprae*. The method developed in this institute and described earlier (⁸) was adopted for separation and purification of *M. leprae*. In short, it consisted of the following steps: homogenization in 0.05 M phosphate buffer, pH 7.0; decontamination with 4% w/v NaOH followed by neutralization with 1 N HCl; treatment with 0.1% w/v each of trypsin, chymotrypsin, and collagenase; exposure to Triton X-100 (final concentration 0.1% v/v) followed by ATPase (final concentration 0.17% w/v, 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 was 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 *M. leprae*.

Bacterial counts. The pin-head method of Hanks, *et al.* (¹⁰) was used to enumerate *M. leprae* in purified suspensions.

¹ Received for publication on 1 July 1983; accepted for publication in revised form on 13 December 1983.

² A. M. Dhople, Ph.D., Medical Research Institute, Florida Institute of Technology, 3325 West New Haven Avenue, Melbourne, Florida 32901, U.S.A.

TABLE 1. Relationship between ATP levels, BI, MI, and viability of *M. leprae* from leprosy patients.

Patient no.	Dapsone treatment ^a (mo)	BI ^b	MI ^c (%)	ATP levels pg/10 ⁶ <i>M. leprae</i>	Mouse foot pad harvests	
					Period (mo)	<i>M. leprae</i> /pad (× 10 ⁶)
1	0	3	24	1.10	8	2.68
2	0	5	19	1.38	11	2.13
3	0	3	17	1.20	8	3.04
4	0	4	15	1.27	11	2.15
5	0	3	14	1.17	8	2.46
6	0	3	13	1.17	11	1.67
7	0	4	13	1.31	8	3.47
8	0	3	12	1.09	8	2.70
9	0	2	7	1.33	11	2.37
10	2.5	4	7	1.24	11	2.28
11	2.5	3	4	0.00	11	0.00 ^e
12	5.0	2	3	0.00	11	0.00 ^e
13	3.5	2	3	1.12	11	1.68
14	2.5	2	2	0.00	11	0.00 ^e
15	10	4	8	0.89	10	1.96
16	10 ^d	2	9	0.94	10	2.46
17	10 ^d	2	7	1.17	10	2.07
18	10 ^d	3	10	0.95	11	2.72
19	10	2	4	0.00	10	0.00 ^e
20	10 ^d	3	2	0.00	10	0.00 ^e
21	5	4	3	0.00	11	0.00 ^e
22	4	3	2	0.00	10	0.00 ^e
23	10 ^d	2	2	0.00	10	0.00 ^e
24	10 ^d	3	2	0.00	11	0.00 ^e

^a Dapsone monotherapy in a dose of 100 mg daily.

^b Bacterial index on the Ridley scale (0–6+) based on three impression smears from each biopsy.

^c Morphological index based on three impression smears from each biopsy.

^d Irregular treatment.

^e Zero values indicate that no AFB were found during the counting procedure. If one AFB is recorded during such counting, the result will be 4.4×10^4 AFB/foot pad; thus, 0 means 4.4×10^4 per foot pad.

Mouse foot pad studies. The method of Shepard (²³) was followed; 6×10^3 *M. leprae* from the purified suspension of each biopsy were inoculated into each hind foot pad of eight normal BALB/c mice for viability testing. For DDS sensitivity of *M. leprae*, mice were divided into four groups; a) no DDS, b) 0.01% DDS, c) 0.001% DDS, and d) 0.0001% DDS. DDS was administered orally, mixed in regular laboratory mouse chow, and fed daily for the entire duration of the experiment. The foot pad harvests were made 8–12 months post-inoculation by the standard methods.

RESULTS

ATP content and viability of *M. leprae*. First, *M. leprae* from biopsy specimens of 24 randomly selected patients were examined microscopically, by ATP assay and by mouse foot pad assay. The results are presented in Table 1. *M. leprae* from Patients

1–9, who did not have any antileprosy treatment, showed a high morphological index (MI, percent of solidly stained acid-fast bacilli) and also contained ATP levels within the normal range of 1.09–1.38 picograms per one million cells. Furthermore, they all gave standard growth curves in the foot pads of mice 8–11 months after the initial inoculations. This suggests that *M. leprae* from all of these patients were viable as well as metabolically active. Among the patients who had dapsone therapy for varying periods of time, *M. leprae* from some patients had low MI, did not show the presence of any ATP, and failed to grow in the foot pads of mice in 10–11 months. These patients can be considered as responding favorably to DDS treatment; the DDS therapy has inhibited the biosynthetic capability of *M. leprae* cells and their ability to grow and multiply and, thus, rendered them nonviable. On the other hand, *M. leprae* from

TABLE 2. Dapsone sensitivity of *M. leprae* from suspected dapsone-resistant patients.^a

Patient no. ^b	<i>M. leprae</i> ($\times 10^6$) per foot pad in mice fed dapsone for 10–11 months			
	None	0.0001%	0.001%	0.01%
10	2.28	1.94	1.39	0 ^c
13	1.68	1.42	0.41	0 ^c
15	1.96	1.38	0.81	0 ^c
16	2.46	1.33	1.20	0 ^c
17	2.07	1.84	1.56	0 ^c
18	2.72	2.13	1.55	0.81

^a Dapsone resistance was suspected from ATP data in Table 1.

^b From Table 1.

^c See Footnote e, Table 1.

other treated patients showed a higher MI, contained ATP in the range of 0.89–1.24 picograms per one million cells, and multiplied at normal rates in the foot pads of mice. In these patients, it can be said that *M. leprae* were metabolically active and, thus, were able to grow and multiply.

DDS sensitivity of *M. leprae* from treated patients. Among the patients who supposedly had been treated with dapsone for varying periods of time but the bacilli from their biopsy specimens still showed the presence of intracellular ATP, DDS sensitivity of these *M. leprae* was determined. As shown in Table 2, *M. leprae* from Patients 10, 13, 15, 16, and 17 multiplied in the foot pads of mice receiving 0.001% DDS but not in mice receiving 0.01% DDS, thus indicating that these *M. leprae* were “intermediate resistant.” On the other hand, *M. leprae* from Patient 18 multiplied in the foot pads of mice receiving even 0.01% DDS, thus suggesting that the bacilli from this patient were “totally resistant” to DDS (¹¹).

Patients 10, 13, and 15 were then taken off DDS therapy, given 600 mg rifampin per day for three months, and were biopsied again. *M. leprae* from all three patients contained practically no ATP and also failed to multiply in the foot pads of mice ten months post-inoculation (Table 3).

DISCUSSION

As mentioned earlier, dapsone resistance has become a serious problem in leprosy endemic areas (^{19, 27}). Until the report of Nath and associates (^{16, 22}) on the rapid, ra-

TABLE 3. Effect of rifampin treatment^a on suspected dapsone-resistant patients.^b

Patient no. ^c	BI ^d	MI ^e (%)	ATP pg/10 ⁶ <i>M. leprae</i>	<i>M. leprae</i> /pad ^f ($\times 10^6$)
10	3	1	0.03	0*
13	2	0	0.02	0
15	4	3	0.00	0

^a 600 mg/day for three months.

^b Dapsone resistance suspected from ATP data in Table 1.

^c From Table 1.

^d See Footnote b, Table 1.

^e See Footnote c, Table 1.

^f Mouse foot pad harvests 10 months after inoculation.

* See Footnote e, Table 1.

diolabeled macrophage culture method for detection of dapsone-resistant *M. leprae*, the mouse foot pad technique was the only available method for determining viability of *M. leprae*. Due to the volume of samples that can be examined, the mouse foot pad method has a number of advantages. On the other hand, it cannot be readily adopted in endemic countries because of financial constraints on maintaining large numbers of inbred mice and adequate temperature controlled rooms. This created an urgent need for a rapid and inexpensive method for the detection of viability and drug resistance of *M. leprae*.

Infectious diseases remain a major cause of morbidity and mortality and yet microbiology has remained a traditional science. One reason is that its tools and techniques are, fundamentally, those developed years ago by Koch, Pasteur, and Gram. Today's common method for estimating viable organisms is colony counting in cultures. A major problem in countering the viability of mycobacteria is the long generation time of the cells, requiring 3–6 weeks incubation time to form colonies. This problem is further magnified when the mycobacterium is *M. leprae*, which has not yet been grown *in vitro* either in liquid or on solid media. Therefore, we adopted the ATP method, using the firefly luciferin-luciferase because of its greater precision, reliability, convenience, and low cost.

The theoretical basis for ATP as a key compound in determining the viability of

TABLE 4. ATP levels, BI, MI, viability, and dapsone sensitivity of *M. leprae* before and after dapsone treatment.

Patient no. ^a	Before treatment				After treatment							
	BI ^b	MI ^c (%)	ATP	<i>M. leprae</i>	Dap-sone treat-ment (mo)	BI ^b	MI ^c (%)	ATP	<i>M. leprae</i> (×10 ⁶) per foot pad in mice fed dapsone for 10 months			
			pg/10 ⁶ <i>M. leprae</i>	per pad (×10 ⁶)				pg/10 ⁶ <i>M. leprae</i>	None	0.0001%	0.001%	0.01%
3	3	17	1.20	3.04	4	3	15	1.17	2.10	1.53	0.85	0 ^e
5	3	14	1.17	2.46	7	3	6	0.00	0 ^e	0 ^e	0 ^e	0 ^e
7	4	13	1.31	3.47	4	4	4	0.00	0 ^e	0 ^e	0 ^e	0 ^e

^a From Table 1.^b See Footnote b, Table 1.^c See Footnote c, Table 1.^d Mouse foot pad harvests.^e See Footnote e, Table 1.

bacteria is well accepted. Dhople and Hanks (3-6) have used ATP as an indicator of growth potential in their *in vitro* cultivation studies on *M. lepraemurium*. The relevance of this assay to the field of infectious disease methodology derives from the ability to subsequently relate concentrations of bacterial ATP to viable bacteria per ml in a biological fluid such as urine or blood, or viable bacteria per gram of tissue. Procedures using this ATP assay technique have been developed for the selective measurement of soluble ATP or mammalian cell ATP in body fluids, and also for the detection and quantitation of bacteriuria and bacteremia (1, 13, 20, 24). Furthermore, Velland, *et al.* (25) have successfully applied this technique to the rapid determination of microbial susceptibility to antibiotics in urinary as well as bone marrow infections. Lee and Crispen have used this technique for the measurement of viable units in BCG vaccine (15) and also for the rapid determination of the effects of tuberculostatic drugs on various pathogenic mycobacteria (14).

The data in our study show that *M. leprae* from untreated leprosy patients contain ATP within a given range, and this range is the same as that observed earlier in *M. leprae* isolated from armadillos infected with human-derived *M. leprae* (7, 8). The results presented here clearly demonstrate that the ATP assay technique can be successfully used to determine the viability of *M. leprae*. The ATP assay results obtained within two hours after receiving the biopsy specimens were further confirmed with the standard

mouse foot pad assay technique, but only after 8-11 months after receiving the biopsy specimens. The cost for ATP assays on one biopsy specimen is less than US\$1; while the cost to obtain the same information (viability) with the mouse foot pad technique is several times greater, not considering the labor and maintenance costs to maintain these inoculated mice for 10-12 months. The results further suggest that even though *M. leprae* from certain patients did not contain any ATP and also failed to multiply in foot pads of mice, the MI did not decline to 0% but varied from 2-4%, indicating that the MI is a less reliable measure of viability. Thus, the ATP assay technique has now made it feasible to obtain information that cannot be obtained by means of successful cultivation. Even given successful cultivation of *M. leprae*, biologic methods will be inferior to ATP determination of viability of *M. leprae* in biopsy specimens of leprosy patients for two reasons: a) *M. leprae* occur in clumps and, therefore, the number of colony forming units will not represent a true number of viable organisms; and b) quantitation of ATP yields practically instantaneous information with much greater precision than the plating method. Our method to extract intracellular ATP from *M. leprae* assures full exposure and total extraction from all of the cells. This method requires very few organisms (1-5 × 10⁶ *M. leprae* per assay). Thus, a standard 5 mm punch biopsy specimen from a patient yields enough *M. leprae* for even triplicate assays.

The experimental design of the present study does not permit a claim that the ATP assay technique can also be used for rapid detection of drug-resistant *M. leprae*, since the dapsone intake by these patients was not under controlled supervision. Thus, it can be stated that the ATP data give information on that status of *M. leprae* at the time of biopsy and that the negative response by some patients to dapsone may be either because their *M. leprae* were dapsone resistant to begin with or because the patients did not follow the treatment regularly. However, the results obtained on three of the untreated patients are encouraging (Table 4). Patients 3, 5, and 7 were put on dapsone treatment (100 mg per day, six days a week) and their compliance to drug administration was monitored regularly by assaying dapsone in their blood and urine samples. All three patients were taking dapsone regularly. Four to seven months after the initiation of treatment, biopsy specimens from these patients were taken for ATP assays and foot pad assays. While *M. leprae* from Patients 5 and 7 lost all of their original ATP and also failed to multiply in the foot pads of mice (even though the MI was 6% and 4%, respectively), *M. leprae* from Patient 3 showed the same level of ATP as before treatment and they also multiplied in the foot pads of mice receiving 0.001% dapsone in their feed. This suggests that *M. leprae* from Patients 5 and 7 were dapsone sensitive, while those from Patient 3 were dapsone resistant. This demonstrates the superiority of the ATP assay technique over the mouse foot pad technique. Immediately upon obtaining the ATP assay results, Patient 3 was put on rifampin and after three months *M. leprae* from this patient did not show any ATP and also failed to multiply in foot pads of mice.

A study is now under way with a large number of patients whose drug intake is being monitored closely to evaluate the potential of the ATP assay technique in detecting drug resistance in a short time period. If this materializes, then the advantage of the ATP assay technique over the ³H-thymidine incorporation method of Nath, *et al.* (²²) is, again, a time factor (no need to incubate for 21 days) and no risk of contaminating samples during such prolonged incubation.

SUMMARY

Mycobacterium leprae obtained from randomly selected lepromatous leprosy patients were used to evaluate the ATP assay technique for detecting viability of these cells. The findings were further confirmed by the standard mouse foot pad technique. While the latter takes about 8–12 months to obtain any valid information on the status of *M. leprae*, the ATP data can be generated within hours and at much lower cost. It is hoped that the ATP data could also instantaneously identify viable bacilli from patients taking dapsone and thereby identify dapsone-resistant patients so that alternative treatment could be given. The advantages of this method over other currently available methods are discussed.

RESUMEN

Se usó *Mycobacterium leprae* obtenido de pacientes lepromatosos seleccionados al azar, para evaluar la utilidad del "ensayo de ATP" como medida de la viabilidad de estas células. Los hallazgos fueron confirmados por la técnica estándar "del cojinete plantar del ratón". Mientras que con esta última se requieren de 8 a 12 meses para obtener información válida sobre el estatus del *M. leprae*, con el ensayo del ATP los datos pueden generarse en unas cuantas horas y a un costo mucho más bajo. Se espera que los datos del ATP puedan llevar también a la identificación instantánea de los bacilos viables en los pacientes bajo terapia con dapsona; esto permitiría la identificación de los pacientes resistentes a la dapsona y su tratamiento con una quimioterapia alternativa. Se discuten las ventajas de este método sobre otros métodos actualmente en uso.

RÉSUMÉ

On a utilisé des cellules de *Mycobacterium leprae* obtenues chez des malades atteints de lèpre lépromateuse et choisis au hasard, en vue d'évaluer la technique des essais par l'ATP pour détecter la viabilité de ces cellules. Les observations ont été confirmées de plus par la technique standard utilisant le coussinet plantaire de la souris. Alors que cette dernière technique exige de 8 à 12 mois pour obtenir une information valable sur le status de *M. leprae*, les données fournies par l'ATP peuvent être disponibles en quelques heures, à un coût considérablement moindre. On espère que les données fournies par l'ATP pourraient identifier de façon instantanée les bacilles viables, chez des malades qui prennent de la dapsona, et dès lors identifier également les malades résistants à la dapsona, de telle manière qu'un traitement de substitution puisse être administré. Les avantages de cette méthode, par rapport aux méthodes actuellement disponibles, sont discutées.

Acknowledgments. This investigation received financial support from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH) (AI-17723); from the THELEP component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases; and from the German Leprosy Relief Association. The author is grateful to Drs. Baliña (Argentina), Opromolla (Brazil), Niemel (Surinam), and Gwinn (NIH, U.S.A.) for arranging to send the biopsy specimens of leprosy patients; to Ms. Barbara Hopp for technical assistance; and to Ms. Beverly Watterson for assistance in preparing this manuscript.

REFERENCES

1. BEUTLER, E. and MATHAU, C. K. A comparison of normal red cell ATP levels as measured by the firefly system and hexokinase system. *Blood* **55** (1967) 311–320.
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. Factors that influence the growth of *M. lepraemurium* in the Nakamura system. *Int. J. Lepr.* **44** (1976) 18–26.
4. DHOPLE, A. M. and HANKS, J. H. *In vitro* growth of *M. lepraemurium*, an obligate intracellular microbe. *Science* **197** (1977) 379–381.
5. DHOPLE, A. M. and HANKS, J. H. Pedigreed stocks of *M. lepraemurium* for cultivation and metabolic studies. *Can. J. Microbiol.* **26** (1980) 1247–1252.
6. DHOPLE, A. M. and HANKS, J. H. Role of sulfhydryls in in-vitro growth of *M. lepraemurium*. *Infect. Immun.* **31** (1981) 352–357.
7. DHOPLE, A. M. and HANKS, J. H. Adenosine triphosphate of *M. leprae*: A brief communication. *Int. J. Lepr.* **49** (1981) 57–59.
8. DHOPLE, A. M. and STORRS, E. E. Adenosine triphosphate content of *M. leprae*: Effect of purification procedures. *Int. J. Lepr.* **50** (1982) 83–89.
9. FIELDSTEEL, A. H. and MCINTOSH, A. H. Effect of neonatal thymectomy and antithymocyte serum on susceptibility of rats to *M. leprae* infection. *Proc. Soc. Exp. Biol. Med.* **138** (1971) 408–413.
10. 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.
11. HASTINGS, R. C. Growth of sulfone resistant *M. leprae* in the foot pads of mice fed dapsone. *Proc. Soc. Exp. Biol. Med.* **156** (1977) 544–545.
12. KOHSAKA, K., MORI, T. and ITO, T. Lepromatoid lesion developed in nude mouse inoculated with *M. leprae*. *Lepro* **45** (1976) 177–187.
13. KULLMANN, L., STAAL, G. E. and WOUTERS, H. W. Increased adenosine triphosphate concentration in erythrocytes in neurofibromatosis. *Clin. Sci.* **42** (1972) 775–779.
14. LEE, Y. S. and CRISPEN, R. A. Rapid quantitative measurement of drug susceptibility of mycobacteria. *Proc. 2nd ATP Methodology Symp.*, San Diego, CA, March, 1977, pp. 219–235.
15. LEE, Y. S., CRISPEN, R. A. and RAISYS, N. R. Measurement of viability of BCG vaccine by analysis of ATP content. *Proc. 1st STP Methodology Symp.*, San Diego, CA, March, 1975, pp. 316–331.
16. NATH, I., PRASAD, H. K., SATISH, M., SREEVATSA, DESIKAN, K. V., SESHADRI, P. S. and IYER, C. A. S. Rapid, radiolabeled macrophage culture method for detection of dapsone-resistant *M. leprae*. *Antimicrob. Agents Chemother.* **21** (1982) 26–32.
17. PEARSON, J. M. H. The epidemiology and some implications of sulphone-resistant leprosy. Abstract in *Int. J. Lepr.* **47** Suppl. (1979) 426.
18. PEARSON, J. M. H., ROSS, F. W. and REES, R. J. W. DDS resistance in Ethiopia. A progress report. *Int. J. Lepr.* **44** (1976) 140–142.
19. PETTIT, J. H. S., REES, R. J. and RIDLEY, D. S. Studies on sulfone resistance in leprosy. 1. Detection of cases. *Int. J. Lepr.* **34** (1966) 375–390.
20. RACKER, E. Bioenergetics and the problems of tumor growth. *Am. Sci.* **60** (1972) 56–63.
21. REES, R. J. W. Enhanced susceptibility of thymectomized and irradiated mice to infection with *M. leprae*. *Nature* **211** (1966) 657–658.
22. SATISH, M. and NATH, I. The uptake of H-thymidine in *M. leprae* inoculated mouse macrophage cultures as a rapid indicator of bacillary viability. Factors influencing the specificity of the *in vitro* assay. *Int. J. Lepr.* **49** (1981) 187–193.
23. 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.
24. VELLEND, H., TUTTLE, S. A., BARZA, M., WIENSTEIN, L., PICCIOLO, A. L. and CHAPPELLE, E. W. A rapid method for the determination of microbialsensitivity using the firefly luciferase assay for ATP. Paper presented at the 74th Annual Meeting of the American Society for Microbiology, Chicago, IL, May, 1974.
25. VELLEND, H., TUTTLE, S. A., SCHROCK, C. A., DEMING, J. W., BAZRA, M., WIENSTEIN, L., PICCIOLO, A. L. and CHAPPELLE, E. W. Application of firefly luciferase assay for ATP to microbial drug sensitivity testing. Greenbelt, Maryland, U.S.A.: Goddard Space Flight Center (NASA), Tech. Rep. (TN D-8439), 1977.
26. WATERS, M. F. R., REES, R. J. W., McDougall, A. C. and WEDDELL, A. G. M. Ten years of dapsone in lepromatous leprosy: Clinical, bacteriological, and histological assessment and the finding of viable leprosy bacilli. *Lepr. Rev.* **45** (1974) 288–298.
27. WHO EXPERT COMMITTEE ON LEPROSY. Fifth Report. Tech. Rep. Ser. No. 607, Geneva: World Health Organization, 1977.