Water-Soluble Complexes of Palmitic Acid and Palmitates for Metabolic Studies and Cultivation Trials of *Mycobacterium leprae*

TO THE EDITOR:

Experimental evidence is accumulating which suggests that palmitic acid might play a major role in the energy-metabolism and probably in the energy-dependent biological processes of *Mycobacterium leprae.* Franzblau ([4, 5]) reported oxidation of palmitic acid with increased synthesis of energy-rich phosphate (ATP) and of phenolic glycolipid-I (PGL-I) by *M. leprae.* Ishaque ([6]) provided direct evidence that *M. leprae* oxidizes palmitic acid via the tricarboxylic acid cycle and the electron-transport chain with O_2 as the terminal electron acceptor. Palmitic acid was more actively oxidized than any other substrate known to be oxidized by *M. leprae.* Since the 16C fatty acid provides close to three times more energy than glucose, palmitic acid should be considered as a candidate energy source in axenic media for *M. leprae.*

Wheeler, *et al.* ([12]) expressed the view that "*M. leprae* requires an exogenous source of fatty acid." As shown by Barclay and Wheeler ([1]), *M. leprae* can indeed hydrolyze lipids and release fatty acyl groups. Fatty acid acquisition from media is therefore an important step in the synthesis of lipids, necessary for multiplication. At least one of the fatty acids might have another role of primary importance: a powerful source of energy. This became evident in the experiments of Franzblau ([4, 5]) and Ishaque ([6]), also supported by the results of Wheeler and Ratledge ([11]), concerning CO_2 evolution when incubating *M. leprae* with palmitic acid.

Palmitic acid and its salts are insoluble in water. These were used in biological systems as a suspension or in liposomes. In none of these conditions is palmitic acid biologically easily available in a solution for mycobacteria.

We are now able to report ([10]) the preparation of water-soluble palmitic acid or palmitate complexes. Heptakis-2, 6-di-O-methyl-beta-cyclodextrin was synthesized and used as host molecules to encapsulate the lipophilic guest molecule, palmitic acid, via molecular encapsulation. The complex is soluble in water, resulting in a crystal-clear solution. When incorporated into biological systems or culture media, dissociation of the complex takes place and continuous release of the molecularly dispersed guest substance is ensured. This unique way of releasing palmitic acid or palmitates results in an improved bioavailability as a prospective energy and carbon source for *M. leprae,* be it for metabolic studies or in culture media. Physicochemical properties of the hydrophilic molecularly dispersed palmitic acid and its salts do not differ from the properties of the hydrophobic structures of the same substances.

In metabolic studies ([1, 4–6, 11, 12]) and cultivation trials ([7]) palmitic acid or Na palmitate was used as a fine dispersion or fine molecular inclusion body, assuming that most microbes can scavenge any substance from a solid state. With the water-soluble formulation of palmitic acid and salts now at hand, this important source of energy and carbon is biologically easily available in solutions for microorganisms, i.e., *M. leprae.* The water-soluble form has the further advantage that the substance is evenly distributed as a solution in an experimental system or a culture medium, rather than as a precipitate or a turbid suspension or emulsion.

The water-soluble complex of palmitic acid or salt is easily incorporated into culture media resulting in clear solutions as in the following example: In 1l distilled water are dissolved KH_2PO_4, 3.5 g; Na_2HPO_4, 2.0 g; $MgSO_4$, 0.01 g; ferric ammonium citrate, 0.05 g; ammonium thioglycolate (60% v/w), 20 ml; Na palmitate solution (10 mg/ml) (Cyclolab), 20 ml. Distributed into screw-cap tubes, the culture media are autoclaved for 25 min. The palmitate is precipitated in the hot media, but completely redissolves as it is cooled to room temperature or kept at +4°C. Dimethylsulfoxide (DMSO) 2 ml was mixed with 18 ml liquid medium and filter sterilized. To each of the 20 ml sterile

media, 0.4 ml of the dilute DMSO solution was pipetted aseptically.

With added 3% agar, semisolid agar slants can be prepared and are free of any precipitate.

One of us (LK) reported that in palmitate-thioglycolate medium seemingly identical, but still unidentified, strains of mycobacteria were regularly grown optimally at +10°C from *M. leprae*-infected human, armadillo and nude mice tissues tentatively named *M. psychrophilum* (L) ([7]). Since the hydrophobic palmitate was replaced by the water-soluble Na palmitate preparation, growth of the above cold-loving strain was easier to detect and follow since the culture was free of undesired, disturbing precipitate as crystals in the media, although the growth was still slow, requiring 8 to 12 weeks for well-developed cultures and subcultures.

We reported the presence of PGL-I in *M. psychrophilum* (L) ([9]). Dr. Enzo Melchior, Jr., Ribierao Preto, Brazil, kindly tested M3 (6th subculture) and A5 (7th subculture) for Mitsuda-type skin reactions in indeterminate, borderline, tuberculoid and lepromatous leprosy volunteer patients. Dr. Melchior found that the heat-killed suspensions (10^7/0.1 ml) of these strains of *M. psychrophilum* (L) gave negative late reactions in all lepromatous leprosy cases (as in the case of the human lepromin). In indeterminate, borderline and tuberculoid leprosy cases, the late reaction was similar to that obtained with human lepromin ([9]).

Cultivation of cold-loving strains of mycobacteria from *M. leprae*-infected tissues is seemingly hard to accept, leprosy being erroneously often classified as a "tropical disease." To regularly find a psychrophilic strain of mycobacteria in *M. leprae*-infected tissues is reminiscent of the observations of Binford ([2]) that "*M. leprae* grow best in the cooler tissues of humans." A similar association between damage from leprosy and temperature was reported by Brand ([3]). These findings raised the question as to whether temperatures cooler than the coolest parts of a human or an animal body are optimal for the growth of *M. leprae*. How cool is cool enough for *M. leprae* to grow faster (or at all) *in vitro* than the well-documented slow growth *in vivo*? Does *M. leprae* not spare the skin fossae, intergluteal regions, and other warmer areas, as shown by Brand ([3])? In cool parts of the body the lesions are heavily parasitized by *M. leprae*, but the proliferation of leprosy bacilli is limited to microscopic lesions in warmer parts of the body ([2, 3]). The thermal difference between normal body temperature and the cool sites is only a few degrees—not more than 4°C. However, this small thermal difference is sufficient to result in heavy growth at the cool site and limited growth at the warm site of the narrow thermal spectrum. These facts suggest that the growth-temperature relation is optimal for multiplication at a temperature much lower than ever recorded in the living body of any of the hosts susceptible to leprosy. Exploration of possible *in vitro* psychrophilic characteristics of *M. leprae* seemed imperative.

The presence of preferably a water-soluble preparation of palmitate and an SH group compound was necessary to obtain the primary cultures and subcultures at +10°C of *M. psychrophilum* (L). The presence of PGL-I in the cultures and the Mitsuda-type late skin reactions with the heat-killed cultures were not sufficient to identify or classify the strongly acid-fast strains. They are, however, "leprosy derived" and due to their unusual characteristics, these findings require a careful follow-up.

With the help of water-soluble forms of fatty acids, palmitic acid and palmitates in particular, further investigations into the cultivation of *M. leprae* are certainly facilitated and invited. These authors offer a limited amount of the water-soluble complex of palmitic acid, Na and ascorbic palmitate to interested investigators.

—Laszlo Kato, M.D.

Catherine Booth Hospital
4375 Montclair Avenue
Montreal, Canada H4B 2J5

—J. Szejtli
L. Szente

Cyclolab Research and
Development Laboratory
59 Pusztaszeri Ut
Budapest, Hungary 1026

Acknowledgment. This study was supported by grants from the Cardinal Leger Institute Against Leprosy.

REFERENCES

1. BARCLAY, R. and WHEELER, P. R. Metabolism of mycobacteria in tissues. In: *The Biology of the Mycobacteria Volume 3. Clinical Aspects of the Mycobacteria.* London: Academic Press, 1989, pp. 37–106.
2. BINFORD, C. H. Comprehensive program for inoculation of human leprosy into laboratory animals. Public Health Rep. **71** (1956) 955–956.
3. BRAND, P. Association between damage from leprosy and temperature. Int. J. Lepr. **26** (1958) 423–424.
4. FRANZBLAU, S. G. Oxidation of palmitic acid by *Mycobacterium leprae* in an axenic medium. J. Clin. Microbiol. **26** (1988) 18–21.
5. FRANZBLAU, S. G. and HARRIS, E. Biophysical optima for metabolism of *M. leprae*. J. Clin. Microbiol. **26** (1988) 1124–1129.
6. ISHAQUE, M. Direct evidence for the oxidation of palmitic acid by host grown *Mycobacterium leprae*. Res. Microbiol. 140 (1989) 83–93.
7. KATO, L. Psychrophilic mycobacteria in *M. leprae* infected tissues. Int. J. Lepr. **56** (1988) 631–632.
8. KATO, L., SZEJTLI, J. and SZENTE, L. Water soluble palmitic acid-cyclodextrin complex in culture media. International Cyclo-dextrin Symposium, Chicago, April 23–24, 1992 (in press).
9. KATO, L., SZEJTLI, J. and SZENTE, L. Water soluble palmitic acid-cyclodextrin complex in media for cultivation of leprosy derived psychrophilic mycobacteria from *Mycobacterium leprae* infected tissues. Acta Microbiol. Hung. (in press).
10. SZENTE, L., SZEJTLI, J. and KATO, L. Solubilization of fatty acids and similar lipids by methylated cyclodextrins. International Cyclo-dextrin Symposium, Chicago, April 23–25, 1992 (in press).
11. WHEELER, P. R. and RATLEDGE, C. Use of carbon sources for lipid biosynthesis in *M. leprae*: a comparison with other pathogenic mycobacteria. J. Gen. Microbiol. **133** (1988) 2111–2112.
12. WHEELER, P. R., BULMER, K. and RATLEDGE, C. Enzymes for biosynthesis *de novo* and elongation of fatty acids in mycobacteria grown in host cells: is *Mycobacterium leprae* competent in fatty acid biosynthesis? J. Gen. Microbiol. **36** (1990) 211–217.