Attempts to Grow *Mycobacterium leprae* in a Medium with Palmitic Acid as the Substrate

TO THE EDITOR:

To use a multifactorial medium under microaerophilic conditions has been proposed by Kato ([4]) in cultivation trials for *Mycobacterium leprae*. Recently, Ishaque ([3]) investigated the effects of various known gas mixtures on the growth of *M. leprae*. An optimal growth, although limited, on both solid and liquid media was obtained when the cultures were incubated under a gas mix-

ture containing 2.5% O_2 and 10% CO_2. No substrate has yet been identified which can serve as a source of energy and carbon for the multiplication of *M. leprae*. Franzblau ([1]) reported evolution of CO_2 during the incubation of *M. leprae* with palmitic acid. Wheeler, *et al.* ([8]) have indicated that *M. leprae* requires an exogenous source of fatty acid. Ishaque ([2]) has provided direct evidence for the oxidation of palmitic acid by *M. leprae*. Based on the effect of classical inhibitors of the electron transport chain, participation of the cytochrome system has been suggested strongly during the oxidation of palmitic acid by *M. leprae*. These studies suggest that palmitic acid possibly could serve as an oxidizable substrate for the growth of *M. leprae*. Attempts were thus made to cultivate *M. leprae* using an optimal gas mixture, i.e., 2.5% O_2 and 10% CO_2, in a medium containing palmitic acid as a substrate.

During these studies, *M. leprae* were isolated from the foot pad lesions of nude mice (athymic) which previously had been infected with human leprosy bacilli. A bacillary suspension was prepared to contain 5×10^8 bacilli per ml. Both liquid and solid media were used. The liquid medium contained $(NH_4)_2\ SO_4$ 0.2 g; $KH_2\ PO_4$, 1.0 g; glycerol 2.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; sodium thioglycolate, 0.4 g; hemin 0.0002 g; sodium palmitic acid, 0.002 g and 100 ml of water. The solid medium, in addition, contained 200 ml of egg yolk.

Several tubes containing 9 ml of the liquid medium were inoculated with 1 ml of the bacillary suspension. To inoculate the solid medium, foot pad lesions were cut into small pieces and a paste was prepared by mincing the tissues in a 2% NaOH solution using a mortar and pestle. Several tubes containing the solid medium were inoculated with 0.3 ml of the bacillary paste. The inoculated tubes were placed in an anaerobic jar. The jar was closed, excess air was removed from the jar, and the cultures were incubated at 34°C in a gas mixture containing 2.5% O_2, 10% CO_2 and 87.5% N_2. Some inoculated tubes containing liquid and solid media without palmitic acid were also incubated under the same gas mixture. The jars were opened every 2 weeks for observation, and the cultures were reincubated. In parallel, some inoculated tubes containing liquid and solid media with and without palmitic acid were incubated as such, i.e., under normal air at 34°C.

The multiplication was evaluated by counting the acid-fast bacilli (AFB). To count the bacilli in the liquid medium, 1-ml aliquots were removed at the time of inoculation (0 hr) and at various time intervals, and the final volume was made to 10 ml with sterile 0.1% skim milk. In order to count AFB from the solid medium, the entire growth from the surface of the medium was removed, homogenized in about 50 ml sterile distilled water, and the final volume was made to 100 ml in 0.1% skim milk. All dilutions made in 0.1% skim milk to count the AFB were sonicated for 30 sec just before staining. This technique resulted in single cells, and no clumping of the bacilli was observed in the stained preparations.

Acid-alcohol-fast bacilli (AAFB) were stained and counted by the method of Shepard and McRae ([7]). In tubes incubated under a gas mixture containing 2.5% O_2 no growth was observed during the first 2–3 weeks of incubation. Thereafter, growth was observed and after 16 weeks of incubation, a sixfold increase in the number of AAFB was obtained. The number of AAFB remained the same up to 20 weeks and then slowly declined. No multiplication of AAFB occurred in a liquid or solid medium without palmitate when incubated under air or the gas mixture. However, it is interesting to note that sixfold increases in AAFB were observed when the cultures were incubated under air for 12 weeks in the liquid medium or solid medium which contained palmitic acid.

The morphology of the bacilli was well maintained. The bacilli did not grow on Lowenstein-Jensen, Dubos, or egg-yolk media. The bacilli recovered from the liquid and solid media grown for 12–16 weeks showed 3,4-dihydroxyphenylalanine (DOPA) oxidase activity, a characteristic specific for *M. leprae* ([6]). Such bacilli also exhibited good endogenous respiration. These observations indicated that the bacilli were, in fact, viable. To further confirm the viability and authenticity of *M. leprae*, the hindfoot pads of two groups of five female nude mice 6 weeks of age were infected with the bacillary suspensions (containing 1×10^7 bacilli) recovered from the solid and

liquid cultures grown for 12 and 16 weeks. In parallel, hindfoot pads of five nude mice were also infected with the suspension (containing 1×10^7 bacilli) prepared from foot pad lesions of nude mice previously infected with *M. leprae*. It was observed that the hindfoot pads of all of the mice infected with the above-mentioned preparations were slightly swollen after about 8 months of infection. Experience has shown that such mice will develop full lepromatoid lesions on the food pads 13–16 months postinfection.

An important aspect of this study is the role of palmitic acid for the growth of the bacilli in the synthetic medium in the presence of air. The use of any gas mixture is quite tedious, time-consuming and laborious. The use of palmitic acid in the presence of air for *in vitro* cultivation trials of *M. leprae* eliminates the use of any gas mixture.

Recently, water-soluble palmitic acid has become available ([5]), and further studies are in progress to compare the role of insoluble and water-soluble palmitic acid in metabolic studies and in cultivation trials of *M. leprae*.

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