Multiplication of *Mycobacterium lepraemurium* in Cell-free Medium Containing α -Ketoglutaric Acid and Cytochrome *c*

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Since the discovery of *Mycobacterium lepraemurium* by Stefansky in 1903, numerous unsuccessful attempts to cultivate it in cell-free medium have been made. This communication may be the first presenting evidence for the unequivocal multiplication of the bacilli in such medium.

The *Mycobacterium lepraemurium* used was the Hawaiian strain obtained from a leproma experimentally developed in the subcutaneous tissue of a C₃H mouse. A bacillary suspension was made with 0.1 % bovine-albumin V (Armour)-saline. For observing the elongation of the bacilli, the method of Nakamura (1967) was used: a drop of the suspension was smeared on a half-size slide, cut lengthwise, and coated with 0.2 % Neoprene W in toluene. After drying, the smeared slide was immediately placed in the culture medium and incubated at 30 °C. After incubation the cultivated slides were fixed with 10 % aqueous formalin, washed well with water and then stained by the Ziehl-Neelsen method.

The culture medium was composed of basal medium and additives. The basal medium, which is referred to as EK medium (enriched Kirchner's medium; Kirchner, 1932), was composed of: KH₂PO₄, 4.0 g; Na₂HPO₄·12H₂O, 4.0 g; sodium citrate, 2.5 g; sodium glutamate, 10.0 g; MgSO₄·7H₂O, 0.6 g; glucose, 2.5 g; sodium pyruvate, 2.5 g; sucrose, 75.0 g; glycerol, 20.0 ml; water to 1000 ml; adjusted with NaOH to pH 7.0. This solution was distributed in 5 ml amounts into test-tubes (10.5 × 1.5 cm), to which was also added 0.1 ml 0.5 % (w/v) calcium pantothenate aqueous solution. The medium was then autoclaved at 115 °C for 20 min. After sterilization 1 ml of bovine-serum, sterilized by pasteurization, 1 ml of 1 % (w/v) α -ketoglutaric acid aqueous solution, sterilized by filtration, and 1 ml of 0.1 % cytochrome *c* freshly prepared aqueous solution, sterilized by filtration, were aseptically added to each test-tube which were then fitted with sterile rubber stoppers. The complete culture medium is referred to as NC medium.

As indicated previously (Nakamura, 1970), significant elongation of the bacilli was observed at the bottom of test-tubes containing EK medium, but no multiplication was seen. However, when a smear of the bacilli was incubated in NC medium the bacilli gradually elongated and consistently multiplied. Finally, the smear was macroscopically recognized as a turbid mass (Fig. 1*a*). Microscopic observation of the smear also showed abundant multiplication of the bacilli after 60 days of incubation. Addition of isoniazid at 10 µg/ml only inhibited multiplication after 60 days of incubation.

A trial for quantitatively counting the increase of bacilli was performed. For this purpose, 0.2 ml of the bacillary suspension, appropriately diluted by 0.1 % bovine albumin V-saline, was inoculated into NC medium, instead of the smeared slide, and the medium was held at 30 °C. At 10-day intervals the culture tubes were shaken with an electric stirrer and the number of bacilli in 0.01 ml of the cultured medium was counted by the method of

Fig. 1. (a) Macroscopic findings of the bacillary smear cultivated in EK medium (left) and NC medium (right). (b) Microscopic feature of the starting material. (c) Microscopic feature of the bacilli cultivated in EK medium at 30 °C for 60 days. (d) Microscopic feature of the bacilli cultivated in NC medium at 30 °C for 60 days.

Shepard & McRae (1968), which is used for counting numbers of *Mycobacterium leprae* in the mouse foot-pad.

The results obtained demonstrated a linear increase in the number of bacilli following a lag phase of 10 days: the initial number of bacilli, per 0.01 ml, was 0.7×10^5 ; after incubation for 10 days, 1.0×10^5 ; after 20 days, 1.5×10^5 ; after 30 days, 3.0×10^5 ; after 40 days, 4.3×10^5 ; and after 50 days, 7.6×10^5 . A possible generation time of *Mycobacterium leprae-murium* can be calculated as 12.3 days.

Multiplications of other acid-fast bacilli, which might have contaminated the starting material, were discounted as no growth of bacilli was recognized in EK medium, which is the most suitable for the growth of acid-fast bacilli.

The bacilli cultivated in the NC medium maintained their pathogenicity to mice. The culture, incubated for 60 days, was shaken by an electric stirrer and 0.2 ml was subcutaneously inoculated into C₃H mice. One hundred days after inoculation the mice were killed and a small leproma was found at the inoculation site. Smears of the leproma, stained by the Ziehl-Neelsen method, showed numerous bacilli which were somewhat elongated with a lot of globi on the smears. No leproma and no bacilli were seen on the smear made from the mice inoculated with a culture of EK medium cultivated under the same conditions as NC medium.

The results show that *Mycobacterium lepraemurium* multiplied in NC medium, which contained α -ketoglutaric acid and cytochrome *c*, and remained pathogenic to a susceptible animal.

Research concerning subculture and biological, as well as biochemical, characteristics of the propagated bacilli is now in progress.

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