

Cultivation of *Mycobacterium leprae*: A New Approach

TO THE EDITOR:

We wish to report our recent findings on our *Mycobacterium leprae* culture isolates-ICRC strains which may throw new light on the problem of cultivation of *M. leprae in vitro* and on the origin of leprosy-derived mycobacteria (LDM). In the course of our investigation on antigenic differences among *M. leprae* culture isolates and on the development of an improved vaccine, we discovered that the ICRC bacilli are not pure cultures, but consist of two mycobacteria with distinctive properties.

Since 1958, in our attempts to grow *M. leprae in vitro*, we have isolated several strains of mycobacteria designated as ICRC bacilli (^{7, 8}). These cultures have been grown exclusively on liquid media, e.g., tissue culture conditioned medium and enriched Dubos' medium (^{8, 21}) (The Table). On the oth-

er hand, the majority of LDM are grown on solid media, and are found to belong to the *M. avium-intracellulare-scrofulaceum* (MAIS) complex. Since some of the ICRC strains isolated during 1958-1961 had shown lepromin-like reactivity, the only marker of *M. leprae* identification available at the time, we continued the work on new isolates with detailed studies on some of the strains (The Table). They were found to share some *M. leprae*-specific characteristics, e.g., lepromin-like activity (⁶), growth in the mouse foot pad (⁵), DOPA-oxidase activity (²²), and antigenic behavior like that of *M. leprae* in mice (^{1, 20}). However, they express a biochemical profile like that of *M. avium-intracellulare* (^{8, 21} and Kato, L., personal communication, 1979). A candidate antileprosy vaccine prepared from one of the strains was found to induce lepromin

THE TABLE. Isolation and cultivation of ICRC and GMC strains.

Period	Medium	Attempts	Suc- cesses	Strains	Authors (Ref.)
1957-1963	TCCM ^a	13	4	C-1, 5, 10	Bapat 1961 (8)
1966-1972	TCCM/Dubos	11	5	C-44	Bapat & Khanolkar 1972
1973-1977	Mod. Dubos ^b	8	6	C-49	Bapat & Modak, 1977 (5,6)
1978-1980	Mod. Dubos	7	2	—	Bapat
1980-1983	Enriched Dubos	10	7	G-75	Kale & Bapat, 1985 (2,21,22)
1984-1989	Mod. Dubos plus processed human plasma	6	2	G-86	Bapat, <i>et al.</i> GMC strains

^a TCCM tissue culture conditioned medium.

^b Mod. Dubos = modified Dubos' medium.

conversion and reversal reactions in LL patients, and has the efficacy and potency equivalent to that of BCG plus *M. leprae* vaccine (10, 12, 13 and Bapat, *et al.*, personal communication, 1980). Experimentally, the irradiated ICRC bacilli were found to induce strain-specific sensitization in mice (20). A similar pattern of sensitization, i.e., differential sensitization to different ICRC strains, was observed in both ICRC-vaccinated LL patients and unvaccinated lepromatous patients (4). The observations summarized above strongly indicated the apparent presence of "*M. leprae*" activity in the ICRC cultures.

The nature of *M. leprae* culture isolates-ICRC bacilli has remained enigmatic for a long time, particularly because of the expression of dual characteristics of both *M. leprae* and *M. avium-intracellulare*. A possibility of "recombination" or "fusion" of two mycobacteria as a mechanism for the phenomenon has been proposed (2). However, the fact that the two sets of enzyme characteristics are mutually exclusive indicates that the mechanism may be remote. An alternate hypothesis of the existence of two mycobacteria in the ICRC culture was presented in 1986 (3). This concept of two populations appeared to be a better and more practical approach to explore the issue.

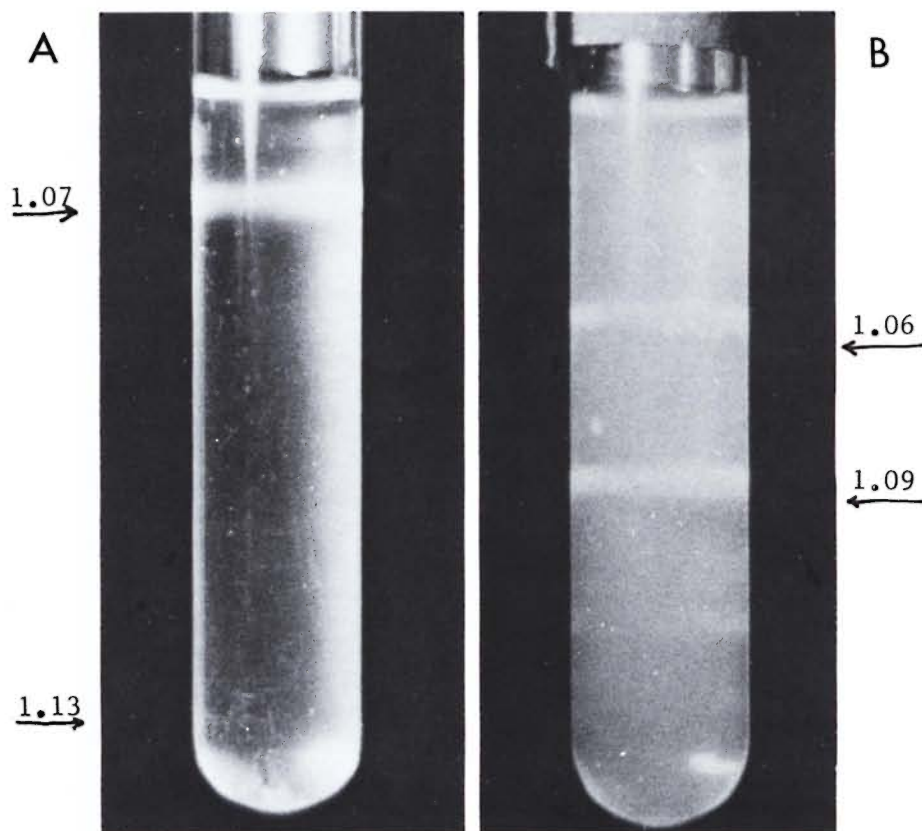
We already know that an inoculum of ICRC bacilli on Löwenstein-Jensen (LJ) medium yields *M. avium-intracellulare* (8 and Kato, L., personal communication, 1979). On this background, we preferred and adopted a physical method—density gradient centrifugation (19)—to search for the other component, if any, in the ICRC culture harvest. The results of the experiments revealed that the long-term *M. leprae* culture isolates-ICRC bacilli did, indeed,

contain just two distinct organisms. This discovery, that the ICRC culture is not a pure strain but consists of two mycobacteria, thus supports the hypothesis (3). By connotation, the discovery has considerable significance and importance for the cultivation problem and the vaccine design. Therefore, we examined the two fractions for *M. leprae*-specific markers. The data collected are presented in this communication, mentioning a few immediate implications.

MATERIALS AND METHODS

Cultures. The ICRC strains (recalled from Dr. L. Kato, Catherine Booth Hospital Centre, Quebec, Canada) and the Grant Medical College (GMC) strains—new isolates—were grown in modified Dubos' medium with human serum (21). A type-strain *M. avium* was grown in Sauton's medium. A few samples of fresh *M. leprae* were prepared from human LL skin biopsies collected aseptically.

Gradient system. A discontinuous sucrose density gradient (5%–30%) was made and approximately 10^{10} organisms were loaded immediately on it. The tubes were centrifuged at $1100 \times g \times 20$ min. The results showed a clear-cut dissociation of the original suspension into two components, the "light" band of 1.07 g/ml density and the pellet of "heavy" organisms of > 1.12 g/ml density. Further enrichment was done routinely. Under similar conditions, on a discontinuous Percoll (19, 27) gradient, the "light" population separated at the density of 1.06 g/ml; the "heavy" population formed a sharp band at the density of 1.09 g/ml. The separation was slow (1 hr 20 min), but the bands were very sharp and stable (The Figure). The results of separation were



THE FIGURE. Separation of "light" and "heavy" populations on density gradients at $1100 \times g$. A = Sucrose, 20 min; B = Percoll, 80 min.

reproducible with all the strains currently maintained as well as with stored samples.

Identification tests. The primary aim was to establish that they were two different mycobacteria. The studies were conducted on two strains, grown in large quantity, and the fractions were subjected to the tests adopted for *M. leprae*-specific markers.

OBSERVATIONS

Properties of "light" and "heavy" organisms

The yield. The proportionate yield of the "light" and the "heavy" organisms was 30:70 on a wet weight basis of the total of 1 g/l at 3 weeks.

Morphology and staining characteristics. Both of the fractions contained acid-fast rods, the majority of which were solidly stained. The "light" acid-fast bacilli (AFB) were slender with uniform spreading; the "heavy" AFB were thicker with few clumps.

Pyridine extraction⁽¹¹⁾ and Ziehl-Neelsen staining. After a 2-hr extraction with fresh pyridine, a slide preparation of the "light" and the "heavy" organisms, along with a fresh *M. leprae* smear as the control, indicated that the "light" AFB lost acid-fastness (also *M. leprae*); the "heavy" AFB did not do so and showed pink rods.

D-DOPA oxidase activity⁽²⁵⁾. The results were reproducible in all of the tests with standardized suspensions, showing that the "light" organisms readily oxidized D-DOPA. They even showed pigment formation on the sediment. The experiments showed $\Delta OD (480 \text{ nm}) = 0.16-0.2$; the activity of the "heavy" AFB was $\Delta OD 0.03-0.06$ as that of the heat-killed bacilli.

Catalase activity. Catalase activity was negative for the "light" AFB and positive for the "heavy" AFB.

Aryl-sulfatase. The results were positive for the "light" AFB and negative for the "heavy" AFB.

Tellurite reduction. Tellurite reduction

was negative for the "light" organisms; positive for the "heavy" bacilli.

Detection of PGL-I antigen (16). An ELISA with polyclonal rabbit antibody to phenolic glycolipid-I (PGL-I) showed a higher concentration of PGL-I on "light" AFB than on the "heavy" AFB.

Growth on LJ medium. The inoculum of "light" organisms did not show growth at 4 weeks; in the same period, the "heavy" AFB produced small colonies of AFB.

Further studies on purified "light" and "heavy" AFB for their lepromin activity, extraction of mycoside, genome size, G+C content, DNA homology, protein profiles of sonicates by SDS-PAGE and by the Amibs protein method are in progress.

Type strain *M. avium*

M. avium bacilli grown in Sauton's medium and placed on the sucrose gradient were found to contain only the "heavy" organisms ($D = 1.12$ g/ml) and no diffuse distribution of any lighter organisms.

Study of fresh *M. leprae* suspension from human skin biopsy. The reproducibility of fractionation with small samples of *M. leprae* culture isolates prompted us to examine the fresh, human *M. leprae* suspension. A 3-ml suspension of minced tissue was filtered through thin cotton mesh, and the filtrate was loaded on the gradient. The organisms separated as "light" AFB ($D = 1.07$) and "heavy" AFB. The "light" AFB in the smear contained many AFB, the majority were beaded and a few solidly stained (original bacterial index 5+), and showed total loss of acid-fastness on the pyridine extraction (PE) test. The "heavy" AFB were few in number and were found to be PE resistant. The paucity of the material and scarcity of the LL biopsies prevented any other tests, except for the inoculation onto modified Dubos' medium.

COMMENTS

The above data show that *M. leprae* isolates-ICRC strains contain two distinct mycobacteria, and this appears to be the characteristic of all (ICRC and GMC) strains grown in liquid media. The evidence supports the concept of two populations as presented in 1986 (3). The properties of the "light" and the "heavy" AFB examined so far establish that they are two distinct mycobacteria, both physically and bacteriolog-

ically. They grow as an established co-culture system, and the dissociation of the two mycobacteria has been demonstrated for the first time.

The "heavy" AFB, the major component from available data, correspond to *M. avium-intracellulare*. The "light" AFB sharing *M. leprae* characteristics, particularly the D-DOPA-oxidase activity at the culture level, may correspond to the "*M. leprae* activity" shown by the ICRC bacilli (22). The final identity status of the "light" AFB will be established when DNA homology data are available.

We believe that detection of a small number of PE-resistant "heavy" organisms in a fresh *M. leprae* suspension may be the key to the question of the origin of LDM. In almost all of the attempts to grow *M. leprae* in culture, when growth is seen it is invariably of mycobacteria belonging to the MAIS complex, irrespective of the medium, the source of *M. leprae*, or the heavy size of inoculum (15, 26). Their presence in the original inocula has been generally accepted (15, 26). From our observations, it is not difficult to imagine a few "trapped" "heavy" organisms in purified *M. leprae* preparations from armadillo-liver tissue which may be the seed of LDM. The AFB in the sediment obtained during *M. leprae* (A) purification appears to have been ignored. The survival and limited growth of *M. leprae* in a variety of special media has been reported with the evidence of growth in the mouse foot pad, but eventually they seem to be swamped by MAIS, and/or eliminated through subcultures (14, 24, 26).

Kato (23) had proposed that "concomitant mycobacteria play a role of an etiological co-factor by providing mycobactin to promote the growth of *M. leprae* in vivo (17, 23). Based on this principle, he has placed *M. leprae* in the category of a microbe-dependent microorganism of the *M. paratuberculosis* type. We seem to have empirically developed a similar system with the ICRC culture, unknowingly using the same concept. This constant yield of "light" bacilli with a growth limit at 3 weeks suggests a mutually controlled growth of the two mycobacteria (8, 15).

The "light" AFB obtained from *M. leprae* culture isolates of the ICRC type, in all probability, could be very close to *M. leprae*. This could have been the component

capable of growth in the mouse foot pad⁽⁵⁾; the source of protein antigens crossreacting with *M. leprae*-derived proteins⁽⁹⁾; and the organisms with epitopes reacting with human T-cells derived from TT patients⁽¹⁸⁾. The responses induced by the ICRC vaccine in LL patients, particularly the reversal reaction mentioned earlier, indicate that the "light" AFB component may be antigenically closer to *M. leprae* than any other mycobacterium. This revelation, that the ICRC bacilli contain an AFB component sharing *M. leprae* antigens mixed with *M. avium-intracellulare*, explains why a single injection of the ICRC vaccine is as effective as Convit's *M. leprae* plus BCG vaccine. The ICRC vaccine preparation inherently embodies exactly the same rationale on which Convit designed his vaccine.

The "light" AFB component appears unable to grow as a single entity. However, the co-culture method and subsequent separation offers a rapid quantitative assay to measure the growth and to evolve a better medium for higher yields of the "light" AFB with other mycobacteria or growth factor such as mycobactin. The practical applications of the system are many: a) a quality control standard to monitor the "light" AFB content of the vaccine batch and to increase potency; b) isolation of pure organisms from a single biopsy source against pooled bacilli leading to the study of strain variations with reference to antigenic epitopes; c) design of a polyvalent vaccine by combination of the "light" AFB from the different strains, e.g., derived from drug-resistant *M. leprae*; and d) to answer many unsolved questions.

In brief, we believe that through the co-culture system yielding ICRC/GMC strains from *M. leprae* isolates a new avenue is opened up to have a fresh look at the cultivation problem. The culture method has been reproducible for many years. It is expected that this will pave the way to solve the most formidable, century-old puzzle in microbiology, namely, the cultivation of *M. leprae* *in vitro*.

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