CORRESPONDENCE

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Has *M. leprae* Been Cultured Already? Response to Kato (IJL 45/2, Editorial).

Hurdles in the way of a mycobacterial strain being accepted as authentic *M. leprae* are many. After a lot of introspection I have been convinced that the biggest amongst them is a mental block of our own making. Through the ages and to date, many claims on cultivation have been disproved. What is disturbing, if not pathetic, is the zeal with which some of us engage ourselves in this task of proving *M. leprae* noncultivable. However, if the block is set aside for a while and the substantial literature reexamined with a clear vision and we recall some of our own experiences and observations as well as those of others, then a trend emerges which is hard to ignore and becomes fascinating and fruitful to reminisce upon.

In the late 1950's Dr. C. K. Becker (IJL **28** [1960] 441-443), essentially a missionary doctor in the then called Belgian Congo, with rather unsophisticated laboratory facilities, obtained a culture which in all probability was *M. leprae*. Lepromatous tissue explants in organ culture grew masses of mycobacteria which he fruitfully used as lepromin. He knew that what he was growing was *M. leprae*. Dr. Wayne Meyers (personal communication) had the privilege of sharing Dr. Becker's experience in his center shortly before Dr. Becker passed away. Dr. Meyers very kindly left with me the few Kodachrome transparencies showing sequential growth of the organisms in tissue culture, and I still possess those old but unique transparencies. Besides the masses of acid-fast (AF) rods, these illustrations also show larger spherical elements in the culture that did not retain the carbol fuchsin stain. As a matter of fact, it was this last phenomenon that I was observing myself in the early 1960's and described as a unique L-form life cycle in *M. leprae* (IJL **33** [1965] 551-555) that prompted Dr. Meyers to produce those Kodachromes in support of my observation. Along with the growth of soft-walled L-form elements of which the AF rods filled spherical masses known as "globi" are the last phase, I also recorded a very small increase in the number of rods of the order of tenfold (10X) over the number used as the inoculum. The inoculum was an enzyme-washed suspension of mycobacteria in lepromatous tissue homogenate, treated successively with pancreatin, pronase and lipase to purify the bacilli. The small increase was not maintained on transfer to fresh medium, but addition of a quantity of lepromatous tissue suspension freed of bacilli and consequently enzyme digested, and subjected to prolonged "battering" in the Raytheon ultrasonic oscillator, did produce a countable increase in number on transfer. Even this stimulation of subcultures faded out on subsequent transfers. This stimulation was not seen if a sonicated homogenate of normal but undigested skin tissue was used. All these are unpublished observations of course, and as stated at the outset, only reminiscences. A yellow-orange pigmented growth was obtained in one of those liquid cultures after seven months of inaction. The medium was rich enough to permit easy and early growth of contaminants if there were any in the inoculum. On subsequent transfers this pigmented organism started growing easily and the time lag for maximal growth became shorter on each

transfer until it became less than a week and on simpler media. This culture was never seriously considered a contender for the honors and was duly forgotten. The reason for nonpursuance was, as mentioned, the mental block and "abiding faith" in noncultivability. These observations, however, convinced me that some unrecognized substance in the tissue was helpful because these experiences of suggestive growth cited here had to do with tissues, autolyzed or enzyme-lyzed, in the milieu. This prompted me to use commercially available powders of testicular, liver, splenic and bone marrow tissues. Well known predilection of *M. leprae* for all these tissues in the human host was of course the rationale. These experiments, however, did not produce results. The now not too famous *M. marianum* grown out of leprous material by Sr. Marie-Suzanne (VI Int. Cong. Microbiol., Rome, 1953, pp 655-656) had a similar evolution: prolonged incubation in autolyzing lepromatous tissue, a yellow-orange scotochromogenic organism, and subsequent adaptation to simpler media and easy growth.

I am tempted to narrate here a story on a lighter vein, but nonetheless instructive. Dr. Carl Taylor was looking for AFB positivity in earlobe clippings of normal individuals in an endemic area in Bengal in the early 1960's. A batch of skin clippings preserved in 2% acetic acid, not by any standard bacteriostatic or bactericidal, was mailed from Calcutta to Baltimore for processing and examination. This batch of 50 biopsies showed an unexpectedly high proportion of positives and positivity of high grade, i.e., location of bacilli was fairly easy and in clusters. Dr. Taylor's speculation that the organisms could have actually multiplied in tissue during transit drew a sharp comment from Dr. Rees (IJL **33** [1965] 716-731) who jocularly suggested that perhaps the way to grow *M. leprae* was to collect biopsies and somehow mail them from Calcutta to take three weeks to reach Baltimore. I am still not sure if these were not actual growths, since I also examined those slides and have not seen anything like them in thousands of other skin clippings I processed and examined subsequently in Calcutta soon after their collection.

The latest report on cultivation from Skinsnes (IJL **43** [1975] 193-203) is, I should say, a refined approach with refined materials, but essentially unable to get away from the familiar trend—only hyaluronic acid replaces crude tissue digests: a refined tissue constituent in the medium, a yellow-orange scotochromogenic mycobacterium, subsequent adaptation to simpler medium and easier growth.

Without much beating around the bush, could we say that once the ice of initial noncultivability had been broken growth became easy. It is possible that hyaluronic acid is a substrate easy for *M. leprae* to handle in initiating more predictable growth away from host tissues, i.e., it is a better substitute to the tissue digests in earlier attempts and unconfirmed successes I have cited. All these sound like bedtime stories and I am quite sure the pundits in the "trade" would instantly brand any such ubiquitous cultivators as eccentric. However, I strongly feel that it is futile to try to characterize *M. leprae* as it will be after it adapts to extramacrophageal existence. It simply does not make sense to try to compare it with Hansen's bacillus that has only known the world of macrophages for generations, I mean its generations. More recent evidence from my own work (Lepr. India **48** [1976] 398-405), and that of Delville (Ann. Soc. Belg. Med. Trop. **55** [1975] 109-118) suggest a dimorphism in mycobacterial isolates of leprous origin, and it seems profitable to try to understand the phenomenon of growth of *M. leprae* from this premise. Both Delville and I have repeatedly isolated a nonacid-fast coccoid organism from leprosy sources which tends to revert to a mycobacterium both in the test tube and in infected mice. Skinsnes in his comments (Lepr. India **49** [1977] 70) on my paper cited above confirms this phenomenon as follows: "In our cultivation effort we grew very slowly and with difficulty for the initial 6-8 weeks, nonacid-fast, small, almost amorphous, coccoid forms. Once this was established, acid-fast rods began to appear as well as some nonacid-fast rods and, in short order, the whole colony became acid-fast and grew vigorously in our medium. Thereafter there was no trouble with cultivating the organism . . . . If we permit the cultures to become old without transfer to fresh medium, or if we transfer our cultures to only hyaluronic acid in phosphate buffer solution, we find the coccoid forms appear and take over with a few acid-fast rods re-

maining. From these we can again grow out acid-fast rods by transfer to fresh medium." Recently two of our nonacid-fast coccoid isolates and one of Delville's have been skin tested along with lepromin in our area on 100 known lepromin reactors and nonreactors. All of the three coccoid antigens produced fairly good early (Fernandez) reaction in all but one of the 14 under treatment and resolved lepromatous cases, and correlated fairly with lepromin. However, none of these produced the Mitsuda reaction in L cases except in one case by the Delville antigen. The immediate tendency is to ignore these findings since many other workers in the past have produced unsubstantiated evidence and had speculated that leprosy was caused by "diphtheroids," as these uncharacterized organisms have always been called. The fact that these coccoid organisms could be one face of the dimorphous *M. leprae* had not been suspected or projected.

I have so far been able to isolate and maintain four yellow-orange pigmented strains grown from leprous material and all these are dimorphous; many others, after initial "viewing," could not be maintained. In old cultures they become nonacid-fast coccoids with occasional acid-fast rods, and on transfer to fresh medium acid-fast and nonacid-fast rods reappear with scattered areas of acid-fast and nonacid-fast coccoids still present. All are scotochromogenic, take about ten days for good growth on plate or slant, have high catalase activity, do not reduce nitrate, and produce niacin or hydrolize Tween 80. In summary, they all fit in the *M. scrofulaceum* complex. This brings us to the crucial issue of establishing the identity of these organisms with *M. leprae,* and it is here that I would like to make a common issue with Dr. Laszlo Kato (IJL **44** [1976] 385-386). He has made a good case for placing test tube grown *M. leprae* in the *M. scrofulaceum* group in the classification scheme. Both he and Dr. Skinsnes have given good evidence of immunologic identity with *M. leprae* of their scotochromogenic strains in specific immunofluorescent staining. However, the only snag is that lepromatous cases have given positive or near-positive reactions (Mitsuda) with the antigens from Kato's isolates (personal communication) and the Hawaii strain HI-75 (IJL **44** [1976] 491-493). If any thing has maintained a consistent trend in leprology, it is the lepromin (Mitsuda) negativity of active L cases. It seems CMI specificity is probably a "little more specific" than serologic specificity. While both the Fernandez and Mitsuda responses are useful the range of responsiveness and their interpretations are different. For establishing specificity of an antigen consisting of *M. leprae* in any form, one would have to be guided by two well documented patterns of responsiveness: 1) Mitsuda negativity in active LL cases, and 2) Fernandez negativity in healthy unexposed adults in a leprosy nonendemic area (acquisition of immunologic experience of *M. leprae* is not difficult, and has to be kept in mind in selecting test subjects). Skinsnes and Kato do not mention the Fernandez response to their antigens. While we have tested our coccoid strains for their skin reactivity, our scotochromogenic mycobacterial isolates from leprous sources manifesting dimorphism have not yet been so tested. But several years ago we undertook a skin test trial comparing antigens of *M. leprae* with tuberculin from *M. tuberculosis* and *M. scrofulaceum.* Response to the former was generally very poor even though tuberculosis is a common occurrence in our area, while the response to the scrofula PPD was very high and almost universal, i.e., in all age and sex groups except for the very young under five years. Incidence of lymphadenitis or scrofuloderma is rare in the area. This observation suggests cross sensitivity to the *M. scrofulaceum* PPD in a leprosy hyperendemic area, and strengthens Kato's hypothesis.

While it is still premature to call these isolates as nearly the same as *M. leprae,* there seems to be enough experimental and circumstantial evidence that calls for more serious interest in these organisms, and studies to establish possible identity or kinship with *M. leprae.* The basis of rejection should not be only a hurriedly done taxonomic matching with known mycobacterial strains. While points of similarity with known strains are important to know, points of difference are more so. An unbiased approach is necessary, and certainly not a hurried one. If Skinsnes' allegation (IJL **44** [1976] 491-493) that conclusion on the Hawaii strain's foot pad behavior was drawn from less than a month of study is true, then it is most unfortunate and quite disturbing. It might still be possible that the search for test tube culture of *M.*

leprae may end in an anticlimax and *M. leprae* may be found indeed to be a ubiquitous organism, cultured already several times in the past, and perhaps Dr. Stanford's progenitors of *M. leprae* (IJL **44** [1976] 216-221) are the cultivable nonacid-fast coccoids, one phase of dimorphous *M. leprae* that some of us are dealing with now.

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