

Approaches to Cultivation of *M. leprae* in a New Laboratory

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This presentation is a résumé of work conducted in the new laboratory of the Leonard Wood Memorial in Cebu, Philippines, during the 15 months since its dedication in February 1964. It represents work in progress and, accordingly, no conclusions can be presented at this time.

The problem of initiation of a new leprosy research program is unique. Probably the most frightening aspect of the task is where to begin. It has now been over 90 years since Hansen reported the presence of a bacterial etiologic agent for leprosy. In the interim a host of competent scientists have contributed to a voluminous literature on the subject. In spite of this fact, knowledge concerning the fundamental nature of leprosy remains in the realm of general acceptance without scientific confirmation.

The bacillus of Hansen, *Mycobacterium leprae*, can be demonstrated in the lesions of lepromatous leprosy with little difficulty. Scarcely a year has gone by, in the past 90, without at least one report of successful cultivation of this organism, and a few of the isolates are even carried in the American Type Culture collection. The size and purpose of this conference, however, attest to the general belief that the causative agent of leprosy has never been grown *in vitro*.

Immunologic procedures have, for the most part, been designed to circumvent this failure in the bacteriology. In the absence of a confirmed etiologic agent, immunologists have resorted to use of pathologically involved tissues containing more or less of the stainable bodies, called *M. leprae*, along with an indeterminate amount of tissue elements. When this confusing status in bacteriology and immunology is considered together with the known long incubation period for the disease, one can

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FIG. 1. Leonard Wood Memorial-Eversley Childs Sanitarium Leprosy Research Laboratory Cebu, Philippines. View of front of the building.

readily see the plight of the epidemiologist. At the end of this biologic sequence, the clinician and ultimately the patient must suffer from lack of knowledge of the natural clinical progression and unavailability of procedures to analyze the effectiveness of new therapy.

In this day of space exploration, it is nothing short of remarkable to realize that the first human disease with which man associated a true bacterial etiology still suffers from such lack of information and so much misinformation. This defective state is obviously responsible for the controversies in which leprosy workers find themselves involved.

In order to bypass this controversy, the research program in Cebu is based only on the primary axiom that leprosy is a transmissible disease, induced by some unknown foreign biologic element. Each hypothesis from this axiom is to be tested on a "yes or no" approach. Accordingly, assuming an infectious agent, we can say that

M. leprae is the primary agent in which we are interested. However, because of lack of knowledge of the possible modes of reproduction of the mycobacteria, we may not rule out the possibility that the *in vitro* morphology and character might differ from that which is observed *in vivo*. We must still accept, also, the possibility that some other primary cause or even secondary modifier may be present. These concepts form the basis for the following research report.

Our inocula, for direct cultivation attempts, were generally human tissue biopsy specimens from lepromatous lesions. They were routinely processed under extensive control to avoid contamination. However, we have also used skin and nasal scrapings, reactional pus, and sternal puncture bone marrow specimens.

In bone marrow samples taken from 100 patients at Eversley Childs Sanitarium, we have observed a high percentage of diphtheroids in stained smears of the specimens.

We were able to isolate the organism, in pure culture, from 37 of the specimens. On primary isolation, in trypticase soy broth, the growth was slow. Typical bipolar stained rods were evident in from one to three weeks. Palisade arrangement and "snapping" distribution characterized the microscopic appearance. On continued laboratory maintenance of the isolates, growth occurred more abundantly and earlier, yielding entire, off-white to cream colored colonies, 2 to 3 mm. in diameter, in 24 to 72 hours on trypticase soy agar. The gram stain varied from negative to positive within single cultures and, interestingly, the gram-positive character increased with age of the culture.

The routine mycobacterial medium in this laboratory is a modified Dubos 20 per cent serum medium buffered in 0.1 M phosphate to a pH of 5.2. When the diphtheroid was transferred to this medium, good growth occurred. The morphology of the organism was altered completely, however. Growth occurred as tight waxy clusters of 20 to 40 coccoid or coccobacillary forms, with diameters of less than 1 micron. These stained gram-negatively in the early growth period, and, as they became progressively older, a few of the coccoid bodies within the cluster increased

in size and became intensely gram-positive. The latter were markedly acid-fast with the Ziehl-Neelsen stain. As the culture aged further, the smaller bodies diminished sharply in number, leaving behind the acid-fast bodies. Eighteen-month-old cultures still showed good viability on subculture, the subculture growth appearing as either rods or coccoids, depending on the medium used.

Intradermal inoculation of living or killed suspensions of this organism into rabbits and guinea-pigs produced equivalent results. An erythematous papule appeared at the site of inoculation within 24 hours and persisted for 3 weeks. No infections resulted from the living inocula, regardless of the route of inoculation. Fermentation reactions and fluorescent antibody tests indicated a relationship, but not an absolute one, among the more than 50 isolations achieved to date. Bone marrow specimens, from 5 persons negative for leprosy failed to yield the organism and all attempts to culture it from the skin of patients and from within the laboratory have failed.

One very interesting observation of this organism was its marked capacity to solubilize the very tenacious pus from a lesion of erythema nodosum leprosum (ENL). The digested pus contained extremely large



FIG. 2. Leonard Wood Memorial-Eversley Childs Sanitarium Leprosy Research Laboratory, Cebu, Philippines, rear view, and quarters for breeding animals.



FIG. 3. Leonard Wood Memorial-Eversley Childs Sanitarium Leprosy Research Laboratory, Cebu, Philippines. View of the general laboratory.

numbers of acid-fast bacteria in dispersed cells, clusters and globiform arrangements. No growth of any kind occurred on routine cultivation attempts with the undigested pus as inoculum. When the digested material, containing both diphtheroid and acid-fast organisms, was inoculated into the low pH serum medium, some indication of an increase in the amount of acid-fast material was evident. This increase was manifested by an augmentation, at 30 days, of 30 to 100 fold in the number of globiform bodies appearing in standardized smears. The globiform bodies appeared as sharply delineated and rounded clusters of acid-fast rods. It seemed unlikely that this increase was due simply to an aggregation of the dispersed rods.

All attempts at subculture of this material have failed. If the subculture was made early enough, there was a limited multiplication of the acid-fast material, but it appeared to represent only a continuation of that amount of activity that was residual in the system at the time of transplant. This would lead one to speculate

that the activity of the primary culture was merely a manifestation of a process occurring within the active, fulminating local ENL lesion at the time of sampling. Comparable experiments in which biopsy tissue preparations were used as inoculum gave erratic results. This seems to be consistent with the contention that the state of the inoculum defined the degree of increase of acid-fast forms.

Another phase of the Cebu research effort that can be reported here was based on the hypothesis that the acid-fast bacteria might have the capacity to reproduce in forms with morphologic and staining characteristics other than those evident in Ziehl-Neelsen stained smears from leprosy lesions. For this work we resorted to use of the QT bacillus, a nerve-invading mycobacterium, isolated by Dr. Binford through hamster ear inoculation with leprosy tissue suspensions. QT grew well on routine mycobacterial media and had the added advantage of behaving as a dispersed "avian" type in broth cultures.

Photometric growth curves of this organ-

ism revealed a consistently reproducible nonlinear fluctuation to the logarithmic phase of reproduction. This fluctuation was rhythmic, imparting a sine wave character of two to three wave lengths to the period of increasing turbidity. Microscopic observations suggested that this pattern was the result of a nearly synchronous variation in the length of the individual bacillary elements. Plate counts supported this contention.

The first microscopic indication of growth was a filamentous elongation of the elements of the inoculum. As elongation occurred, cross striation also was evident with dark field microscopy. The long, cross-striated cells stained with the acid-fast stain as sharply beaded units, only the cytoplasmic material retaining the stain. It must be stressed, however, that in dark field microscopy, the cell walls appeared as firm, sharply refracting and apparently healthy elements. During the period of photometric senescence, the longer filaments became fragmented into shorter units. This intermittent fragmentation was assumed to be responsible for the intermittent reduction in the rate of reproduction, as determined photometrically. Viable growth curves of the same cultures could be plotted as linear. It is possible, however, that this less sensitive procedure did not lend itself to the demonstration of these discrete changes.

At 30 to 40 days, in this medium, the QT bacillus reached the end of the stationary phase of growth and went into a long period of slowly declining turbidity. This period was marked by the disappearance of the filamentous form and an increase in numbers of short solidly staining acid-fast bacilli.

This pattern of morphologic variation from long beaded filamentous to short solid organisms, was related to the character of the inoculum. As the cultures aged, they tended to stabilize in the short solid form. Continued subculturing of these older cultures progressively shifted

the population of the early growth phase in favor of the short solid form, and finally the filamentous form was no longer evident. Late transplants grew as short solid forms over the entire growth curve. Concurrently with this shift, the culture appeared to lose its capacity to grow on Loewenstein-Jensen medium. Massive inoculations of the Loewenstein-Jensen medium, however, after a relatively long lag phase, yielded a few white, viscous colonies. These colonies were filamentous and, interestingly, also predominantly nonacid-fast. With aging, and also on subculture in the acid serum medium, the acid-fast filamentous form again appeared.

SUMMARY

We have been able to isolate diphtheroids of related character from a significant number of leprosy patients. Investigation of these organisms showed a pleomorphism that was associated with the conditions of growth. The diphtheroid produced no infections *in vivo*, but did produce interesting skin test reactions. Reactional pus, containing *M. leprae*, when digested by the diphtheroid and incubated in a suitable medium, yielded primary cultures that gave evidence of increasing numbers of acid-fast bodies. The mycobacterium QT gave evidence during growth of a transition from long filamentous to short solid forms. This conversion appeared to represent a stabilizing transition and to be related to age and conditions of growth.

The observations reported here are on incomplete research in progress. It is felt that any conclusions at this time would be premature.

Acknowledgments. I would like to close by noting that this research is a joint venture of the Leonard Wood Memorial and the Department of Health of the Philippine Government, and receives financial support from Grant AI-04809 from the National Institute of Allergy and infectious Diseases, National Institutes of Health, Bethesda, Maryland. I also acknowledge the assistance of Dr. David A. Power and Dr. Rodolfo Abalos in this effort.

DISCUSSION

Dr. Cochrane. This is all very interesting to me. I would like to draw attention to the work of Dr. John Reenstierna about 30 years ago. He described nonacid-fast diphtheroids that changed into rods in his studies on leprosy. Part of his account was reprinted in the *INTERNATIONAL JOURNAL OF LEPROSY* in 1938 (**6** (1938) 77-90); a good deal of it came out in the *Acta Scandinavica*. I think it would be very useful if Dr. Reich went back to some of these old workers. It makes us realize that there is really nothing new under the sun.

Dr. Goldman. Dr. Reich showed a symbiotic relationship between an unidentified organism and an acid-fast organism in culture. He obtained this unidentified organism, I believe, from marrow, and grew the acid-fast organism in the presence of the symbiont. The symbiont seemed to disappear after two, three, or four weeks of culture. I would like to know if the symbiont will grow by itself or if it requires the presence of an acid-fast organism, and also if the symbiont has any pathogenic or pathogenic characteristics itself.

Dr. Reich. We called the symbiont a diphtheroid originally. Now we call it X

for want of a better name. The morphologic variants of X will grow very readily, and independently, on agar medium. We shifted its form from the Ziehl-Neelsen gram-negative stage to the gram-positive, Ziehl-Neelsen-positive stage by transferring it from Trypticase soy broth to serum broth. We had also prepared a fluorescent antiserum against the killed form of X by the time I left, and a letter received since I arrived here informed me that we now have a fluorescent antiserum against the live form. We have injected the killed form, the live form, and lepromin intracutaneously into animals in an attempt to evaluate the relationship between the skin reactivity of X and the lepromin test. We have found no correlation. However, the killed and the live organisms when injected intracutaneously, caused no apparent involvement, but a papule was raised at the site of inoculation. It appears about 24 to 48 hours after injection and persists for 3 weeks before the site returns to normalcy. We also injected the living organisms intravenously, subcutaneously, and intraperitoneally, and, up to the time I left, there was no evidence of any pathogenic effect. We used rabbits and guinea-pigs as experimental animals.