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The Cultivation of *Mycobacterium leprae* Search for a Rational Approach[1]

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It has always been recognized that the problem of cultivating *M. leprae* is the bottleneck in essentially every avenue of leprosy research. Nevertheless, we may represent the first occasion when a thoughtful group of biologic and medical scientists has congregated in recognition of this fact. It seems to me that this occasion has an even deeper significance. It acknowledges also that the great flourishing of basic knowledge of microbial cytology and physiology has attained a useful maturity. At last the microbiologist is in a position to analyze the elements in such problems and to contribute toward their solution. I wish, therefore, to combine congratulations with my personal thanks to all who have joined in this series of illuminating discussions.

My own role will be to summarize the seemingly instructive aspects of recent investigations and to describe for your criticism the sign posts that might guide further work on the cultivation of *M. leprae*.

In the early work by myself and associates a rational approach was delayed by several assumptions that proved to be erroneous, viz., (1) that the special requirements of host-dependent microbes can be met by cells, metabolites, or components of host systems, (2) that the cultivable mycobacteria might provide reliable examples of pH optima, physiologic requirements, respiratory mechanisms, etc., and (3) that the dependent mycobacteria divide by binary fission, producing cells with rugged and relatively impenetrable cell walls.

During the period 1939-1959 the pertinent disciplines of medical microbiology were applied with innovations hoped to be useful, but without driving a single entering wedge. Twenty years is sufficient for learning what not to do. The choice was either to quit or to replace mental evolution with revolution and to seek a new basis for learning.

The propositions were elementary: that the unique problems of microbes are related to membranes that synthesize cell walls, and that one should focus upon competent microbes, not hosts, as the source of specialized cofactors.

Happily, the mycobacteria are so diverse that a relatively unexplored species, *Mycobacterium johnei*,[3] fitted several interesting requirements. Since this species requires mycobactin for *in vitro* growth, it was thought to be strongly host-adapted. It does not produce lesions in pulmonary tissues. If deprived of mycobactin, cells in the resultant noncultivable states, at least in some respects, should afford counterparts of the problems in the noncultivated species. It is the most fastidious type that can be subjected to study by all of the laboratory disciplines.

Since 1960 one segment of our work has focused on three strains of *M. johnei* and two strains of wood pigeon mycobacteria, which have similar chelate requirements. Events have shown that all are cut from the same piece of cloth, and I shall simply use the term chelate-requirers in describing the results of this work. Studies with these unconventional models have opened our eyes in many directions, particularly as regards their affinities with soil microbes and their inability to cope with major features of host environment.

SIGN POSTS DURING THE PAST FIVE YEARS

The character of chelate requirements. Finding ourselves enmeshed in the diffi-

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culty of mycobactin production and titration, we learned first that special requirements for microbially synthesized chelators of heavy metals have not been imposed upon microbes by prolonged adaptation to cows, sheep or birds. Several species of free-living bacteria and fungi also have similar requirements. Reich and I [15] took advantage of this relationship by using the soil microbe, *Arthrobacter terregens*, to devise a mycobactin bioassay, which was later improved by Antoine, Morrison and Hanks [1].

Rypka and Antoine (unpublished observations) observed that withholding iron from the mycobactin-synthesizing saprophytes increases the production of mycobactin. Further studies by Dhople and Morrison (unpublished observations) have demonstrated that the membranes of cells grown on limited iron amounts are deficient in cytochromes. This raises interesting questions, viz., if an alternative pathway transports electrons to oxygen, and if such pathways are of major importance for host-grown mycobacteria.

After concluding studies on the role of autoclave factors in circumventing the mycobactin requirements of *M. johnei* [8], Morrison, Antoine and Dewbrey [9] conceived the idea that the essential property shared by a long list of microbially synthesized chelators is the formation of relatively nonpolar chelates. By switching from the policy of natural feeding to that of bottle-feeding, they were able to stimulate the growth of *A. terregens* by means of acetylacetone, salicylaldehyde, 8-hydroxyquinoline and similar "poisons."

The metabolic systems that require supplementation by nonpolar chelates remain to be defined.

The character of host dependency. We have seen that the most fastidious of the cultivable mycobacteria resemble soil microbes in their major dependency upon more competent microbes. This has raised interesting questions regarding the possible role of animal hosts as conveyors of strictly microbial growth factors and the intracellular utilization of such factors. Exploration of these possibilities has permitted us to define the imprint of host adaptation and forced us to conclude that the major genetic markers in the models studied could not have been selected by prolonged existence in mammalian environments.

The essential findings have been as follows:

1. In the presence of mycobactin, and irrespective of pH, the efforts of Reich, Wheeler, Power and Hanks to improve growth by means of host derivatives and peptones have produced more inhibitions than stimulations. One of the most interesting observations is that peptones in 0.2 per cent concentration provide a factor that assists in reducing the lag prior to growth, does not increase total cell crops, and is particularly important in media of low osmotic pressure.

2. The first cells suitable for nutritional and physiologic studies were produced as a result of Morrison's [8] investigation of the circumvention of mycobactin requirements during the growth of pellicles on the Watson-Reid synthetic medium. This work demonstrated the marked inferiority of filtered media, the importance of autoclaving and inoculating the medium at pH 5.5, and the fact that circumvention depends upon the formation of autoclave factors. It was shown also that the synthesis of new cells requires merely the NH_4 ion, fumarate or malate, and glucose and glycerol.

3. In other studies, Wheeler and Hanks [20] used diluted suspensions of declumped cells in order to study parallelisms between intracellular and extracellular growth. Particular interest was attached to the finding that mycobactin, iron, CO_2 or glycerol, which permit or promote the growth in the WR medium, stimulate also the growth of bacilli that are secluded in mammalian cells. The dramatic influence of mycobactin on intracellular growth represents a new phenomenon, the intraphagosomal use of strictly microbial growth factors. Since there is equally clear evidence in the literature [5] that lysozyme and host globulins gain access to phagocytic vacuoles, it may be concluded that the nutritional situation for intraphagosomal microbes is not fundamentally different from that of the extracellular types; also that the biochemical

environments in different tissues can be important determinants of intracellular growth.

4. Further studies in bacteriologic media have demonstrated that the optimal pH range for initiating growth is 4.5-5.5. Using the optimal synthetic medium neither Wheeler and Power nor I have been able to find host components that will eliminate the lag prior to growth. We have shown, however, that definite advantages are gained by simplifying the usual complex media.

At this point we have described mycobacterial pathogens with a fixed adaptation to the physiochemical environments existing within phagocytic vacuoles and the genetic incapacity to become adapted to major features of host environment, i.e., the pH and proteins of the body fluids and the pH and complexity of cell cytoplasm. Similar genetic straight jackets do not exist in brucella or in bovine type tubercle bacilli that might be living within the lymph nodes of the same cow. There seems no point in arguing that the peculiarities of the chelate-requiring mycobacteria were acquired because of prolonged intracellular parasitism. The requirements for siderамines are shared with a series of soil microbes. Requirements for low pH are common among yeasts and fungi. A preference for the NH_4 ion, the need for an iron-bearing growth factor (heme), an intolerance to conventional levels of peptones, and stimulation of growth without increase in cell crops, are major markers in certain of the cultivable rumen bacteria ([3]).

To summarize as briefly as possible, the chelate-requiring mycobacteria are best regarded as soil microbes that attract our interest because they have found a haven in the phagocytic vacuoles of cows, sheep and birds. Whether they exist there by circumventing mycobactin requirements or whether they live adjacent to the gut and to the portal circulation of the liver in order to borrow microbially synthesized growth factors, is an interesting question for the future.

The imprint of host adaptation. The foregoing views do not contradict the classical observation that microbes are modified by prolonged existence as parasites. When similar inoculums are used, a major discrepancy is noted between the prompt growth within tissue cells and the lag periods of 5-6 weeks prior to growth in the WR medium containing mycobactin. This indicates ineptitude in saturating *in vitro* environments with metabolites and cofactors. Although these excessive periods of lag have not been relieved by host components or hydrolysates, they are readily eliminated by either of two principles: the "lend-lease" of microbial factors, and the use of viscid menstrua to impound slowly synthesized cofactors near cell surfaces.

As shown in Figure 1, the 5-6 week lags in onset of growth have been eliminated by supplementing the WR medium with supernates from cultures of competent mycobacteria and also by merely adding 20 per cent gelatin to the WR medium. The latter principle was used by Necas ([11]) and by Landman and Halle ([7]) to promote the mass conversion of the protoplasts of yeast and of *Bacillus subtilis* to complete cells. It is analogous to the enclosure of microbes within phagocytic vacuoles.

The synthesis of cell walls. During the early stages of these studies, we were greatly impressed by evidence that the cell walls on the noncultivated *M. lepraemurium* and *M. leprae* are at least two orders less penetrable than the walls on saprophytic species ([6]). While this is true of the most rugged members of mycobacterial populations prepared by conventional methods, experience has shown that as one crosses the spectrum from saprophytes to noncultivated species, the synthesis of walls becomes increasingly difficult. In synthetic media containing 1-3 per cent Tween 80, both *M. phlei* and the attenuated tubercle bacillus R1Rv can attain maximal rates of growth in solutions providing an osmotic pressure equivalent to approximately 1 mammalian iso-osmole ([13]). The cells of *M. phlei* are flexible and enlarged, while those of R1Rv encounter a period of osmotic sensitivity that interrupts growth. Even in the absence of Tween 80, strain Mj68 of *M. johnei* becomes osmotically sensitive in 1.8 iso-osmoles (Wheeler and

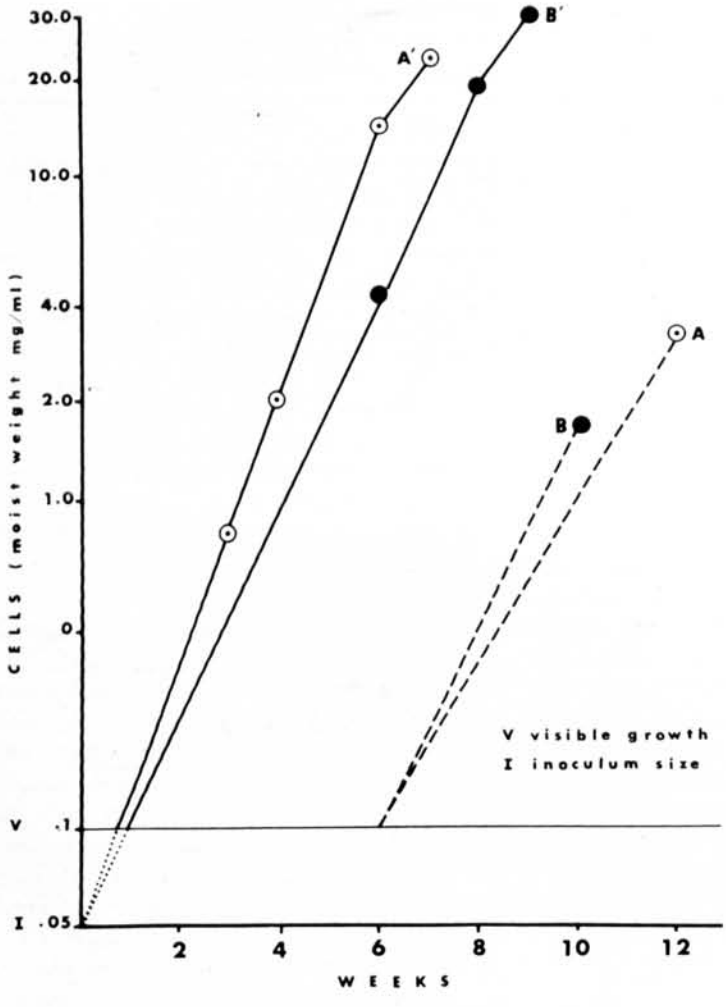

FIG. 1. Elimination of lag prior to the growth of *M. johnei* strain Mj68 in the Watson-Reid synthetic medium. ○ = Wheeler and Hanks, unpublished observations. ● = Power and Hanks, unpublished observations.

Hanks, unpublished observations). In media of 3.5 iso-osmoles and including 1 per cent Tween, growth may occur solely in the form of spheroplasts ([13]). As emphasized by Chatterjee, *M. leprae* grows in lepromatous patients as spheroplasts and transitional L forms. As shown by both Chatterjee ([4]) and Reich ([14]), a major factor in the limited multiplication of *M. leprae in vitro* is the difficulty in formation of cell walls.

SIGN POSTS FOR THE FUTURE

From the descriptions of Chatterjee ([4]) and Reich ([14]) several portents for the future have become evident, viz.: (1) The long-sought entering wedges for direct study of the propagation of *M. leprae* have been discovered. (2) *M. leprae* exhibits a marked dichotomy between the synthesis of subcellular units and the construction of complete rods. (3) Because of the great variety of growth forms, microscopic methods do not provide critically needed information on two points: (a) the total replication of genetic units and cytoplasm, and (b) the relative success in the formation of cell walls.

Any inspiration regarding useful methods should have high priority over further exploration by present methods. Although the amount of fluff which now accumulates in Chatterjee's cultures would encourage one to believe that characterizations could be undertaken by chemical or bioassay procedures, the excessive periods of incubation and the difficulty of having material to prepare the necessary numbers of

replicate cultures leave us in a quandary.

General policy. It may be expedient to proceed a bit further with microscopic methods while exploring several existing principles and possibilities. Before proposing to deal with possibly deep seated problems in the production of energy, a first consideration is to protect, activate, or supplement all operable systems. The principles elucidated in studies on the chelate-requiring mycobacteria include osmotic protection by high concentrations of amphoteric electrolytes, efforts to impound near cell surfaces those components that a microbe may synthesize too slowly, and loaning of the multiple factors that have been synthesized by competent mycobacteria.

Synthesis of cell walls. In view of the problems in *M. leprae*, I am particularly grateful for the discussions by Nickerson ([12]) and Ulrich ([18]) on the modulation of cell wall formation in yeasts and fungi. From the contributions by Moulder ([10]), Chatterjee ([4]), and Reich ([14]), it is evident that delayed and incomplete cell wall formation are prominent symptoms of the deficiencies in host-dependent microbes. Although this indicates problems in membrane function, it might be useful to consider a reversal of this logical argument. When microbes with faulty membranes are removed from intracellular environments, the usual conditions *in vitro* require that they saturate much larger spaces with metabolites and cofactors. A possibility exists that extracellular losses by dilution include not merely the conventionally recognized soluble factors but also wall precursors and even critically limited components from their energy-capturing systems. It will be recalled that even the most competent of microbes are handicapped in respiration and synthesis in the absence of cell walls. It seems possible, therefore, that assistance in wall formation might result in the construction of more competent membranes.

Specific lists of the monomers required for wall formation in mycobacteria are available ([2,16,17]). As polymerizing agents we have both *M. leprae* and the chelate-requirers that can be grown as spheroplasts. Since the latter undoubtedly possess both templates and adequate energy-generating systems, it seems that we have a model in which to investigate whether slow wall formation could be due to the slow saturation of environments and cells with the known monomers, and major gains can be made by supplying larger complexes. One may imagine, for example, that complexes such as mycolic acid, containing 88 carbon units, might not be synthesized readily.

Problems in energetics. This problem is not discussed last because it is considered to be least, but because not everything can be undertaken at once. The universal production of nonpulmonary lesions by *M. lepraemurium* and *M. leprae* suggests that it might be wise for all of us to acquire the ingenuity or equipment to conduct certain segments of any work at minimal concentrations of oxygen.

Meanwhile, we may hope to see certain questions clarified. Tubercle bacilli and *M. lepraemurium* grow *in vivo* without forming cytochromes in amounts that have been measured. Severe deficiencies also have been produced in saprophytic species by growing them in the presence of azide (Dhople and Morrison, unpublished observations), and possibly by the selection of INH-resistant mutants (Mifuchi and Morrison, unpublished observations). We may have models therefore, in which to analyze the possibly important use of alternative pathways during growth under conditions of limited iron or oxygen. There remains also the problem of determining whether the role of mycobactin is related to the cytochrome pathway, to alternate pathways, or to other systems.

Given a clearer understanding of the pathways whereby mycobacteria can gain energy, the prospects for bold innovation have been brightened by an increasing body of evidence, as summarized by Moulder ([10]) and Weiss ([19]) that external supplies of cofactors can be utilized in electron transfer and the capture of energy.

For those of us who are concerned with the noncultivable mycobacteria, there remains the important question of experimental material. One dilemma is that we cannot yet prepare pure suspensions of *M. leprae*, and that we cannot obtain sufficient

cells to proceed by metabolic analysis of the effects of supplementing individual systems. It seems, therefore, that we may be forced to measure over-all benefits by cytologic and biologic methods.

SUMMARY

I have undertaken to outline a few of the conceptual road blocks that have delayed comprehensions and analysis of the multifactorial problems involved in the cultivation of *M. leprae.* A remarkable flourishing of knowledge of the physiology and cytology of microbes, combined with specific work in this field, seems to have permitted more useful views in several directions. Aside from operational knowledge, tangible gains have been made by:

1. Recognizing the inadequacy of host-oriented concepts.

2. Demonstrating that, except for physical environment, the nutritional situation for intraphagosomal microbes is analogous to that of the extracellular types.

3. Realizing that problems of delayed or defective wall formation occur in all host-dependent microbes; that hosts may not make or use certain of the constituents; and that microbes are the logical source of the unique monomers, templates and supplements required.

4. Appreciating the coincidence between strictly nonpulmonary infections and special problems in the mycobacteria that cause them.

5. Knowledge of the cytochrome-deficient states in host-grown mycobacteria, by suspicion that they may rely heavily upon alternative pathways, and by increasing ability to supplement energy-capturing systems.

6. The possession of an entering wedge with *M. leprae* and by having models in which to test possibly useful principles.

I feel, therefore, that the major sign posts at least for specific avenues for further work have been recognized. We are left with at least two classes of questions: (1) the possibility that other clear sign posts have been ignored, and (2) serious problems of strategy when multifactorial balances must be achieved simultaneously. Finally, I am particularly grateful for the opportunity to hear these considerations discussed by persons with such diverse and extraordinary insights into related problems.

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DISCUSSION

Dr. Rees. I am sure that I voice on behalf of all of you many thanks to Dr. Hanks for his wise and stimulating talk, which in many ways summarizes the questions and ideas we had hoped, perhaps not too successfully, to raise during the earlier part of this symposium. Undoubtedly many of these points are highly relevant and if we are to make progress must be tackled now by direct studies of *M. leprae*.

Perhaps because I came into the leprosy field after first working in another discipline, I must admit that, although I have been stimulated by the suggestions that the bacillary form of *M. leprae* may be only one part of its "life cycle," I am worried about the basis on which this hypothesis has been presented. Because in the past the field of leprosy research has been limited by failure to culture the causative organism or to transmit the disease to animals, leprosy has fallen prey to many different, and often unscientific hypotheses regarding etiology, etc. Although *M. leprae* can still not be cultured, there is worthwhile evidence that it can be transmitted to animals, and already at this conference it has been revealed that this advance is providing important basic knowledge. Now that, for the first time, direct studies based on sound principles, can be undertaken, I fear that the "life cycle" hypothesis may put the clock back unless the work is tackled objectively and all the past pitfalls are appreciated. The particular points I feel require special consideration are: (1) Because *M. leprae* can be obtained only from homogenized tissue, evidence of "L forms" based on morphologic appearances alone must be suspect because of the possibility of artifacts; (2) the known ease of isolating cultivable strains of mycobacteria and other passenger bacteria, especially diphtheroids, from skin biopsies; and (3) the availability of the foot pad technic, tissue culture, and lepromin test for determining the identity of *M. leprae*. Now as your chairman I have the problem of deciding what to do at this late hour. Having heard the particularly deep and wide range of Dr. Hanks' suggestions, I ask you—do you want this thrown open for discussion or do you want to cogitate?

Dr. Dharmendra. References have been made to my statement this morning, and I am afraid that the main object of my statement has not been understood. What I objected to, and still strongly object to, is the confusing terminology of *M. leprae muris* and of rat leprosy. *M. leprae muris* is not a variant of *M. leprae* and as it is seen in rat leprosy is not leprosy as seen here.

Dr. Rees. Yes, Dr. Dharmendra, I do not think any of us would hesitate in agreeing with you about the terminology of the organism. One can't help thinking at this stage, of course, that the whole of leprosy is contaminated by terminology and classification, but I would hope you didn't mean that, from a scientific point of view, we should be directed on what models we use and what models we don't use.