THE ORIGIN AND CHARACTER OF NONCULTIVABLE STATES IN MYCOBACTERIA [1]

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One of the goals of scientific study is to define and analyze problems in such a way that logical experimentation can lead to their solution. While it is true that rat and human leprosy bacilli cannot yet be cultivated on bacteriologic media, it is now possible to define the character of the cultivation problem. Two factors stand out: (a) the exceptional impenetrability of these bacilli, and (b) certain peculiarities in their respiratory systems.

Encouraging leads are afforded by study of the related, factor-requiring mycobacteria. These bacteria readily adapt to growth without added factor if their environment approximates that in phagocytic vacuoles of host cells. Given mycobactin, or other appropriate conditions, they retain the complete synthetic capacities of saprophytes and tubercle bacilli. They profit little, if any, from complex nutriments. In brief, they do not show a trend toward dependence on materials or enzymes within host cells. Finally it is of particular interest that each factor or condition promoting growth in tissue cells promotes growth *in vitro* also. Since the converse is true, there are strong indications that any point established in either of these biological systems will have a general validity.

Evidence is presented in this paper that knowledge of the cultivation problem has advanced in three directions, through (a) definition of basic questions, (b) recognition of the value of studying species intermediate between tubercle and leprosy bacilli, and (c) understanding of a common significance among biochemical, nutritional and cell culture methods used in their study.

THE ORIGIN OF NONCULTIVABLE STATES

Noncultivable states seem to originate from excessive development of two properties shared by all pathogenic mycobacteria, viz., relative impenetrability and minimal capacity for respiration.

Impenetrability.—Of all microbes, the mycobacteria are genetically richest in lipids. Pathogenic species resist digestion within host cells because of a synthesizing cord factor, sulfolipids and hard waxes in their composition. Rat and leprosy bacilli are the least penetrable of all species ([2]). The following important questions are related to this exceptional impenetrability: Does it arise solely from selection for resistance to host enzymes, or, in part, because carbon is used as an acceptor of hydrogen and electrons, i.e., to compensate for deficiencies in respiration? Is it a major impediment to the *in vitro* transport of elements essential to growth?

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Respiratory deficiencies.—It seems curious that the strictly aerobic mycobacteria should be able to proliferate within mammalian tissue cells, which are facultative anaerobes. It has long been evident, though not fully appreciated, that the pathogenic mycobacteria fall into two groups with respect to oxygen tolerance in host cells and tissues, as indicated in Table 1. Some kinds of mycobacteria cause infections in pulmonary or other ventilated tissues. These grow readily *in vitro* and in cell cultures. In contrast, many mycobacterial infections are strictly nonpulmonary. This fact indicates clearly that, even within the complete host, the causative agent proliferates poorly when host cells are exposed to modest concentrations of oxygen. Without exception, the causative agents either require special growth factors or have not yet been cultivated.[2]

TABLE 1.—*Grouping of mycobacteria in relation to (A) ventilation of infected tissues and (B) cultivability.*

(A) Natural sites of infection	None	Usually pulmonary Tbc		Nonpulmonary		
Mycobacteria		Av.	Hum. Bov.	*M. johnei*	*M. lepraemurium*	*M. leprae*
(B) Cultivability In tissue cells.	—	+	+ +	+Mb+CO²[a]	+	—
Independently	+ + + +	+ + +	+ + +	+Mb+CO²[a]	—	—
Other types or sources of mycobacteria		Atypical and anonymous Respiratory tract and skin lesions		Scandinavian wood pigeons[a] Water buffalo, D.E. Indies Mexican green parrots Pacific salmon Bolivian bullfrogs		
				Encapsulated lung lesions Cutaneous tuberculosis Mycobacterial ulcers		

[a] *M. johnei* and the mycobacteria from Scandinavian wood pigeons are similar Mycobactin (Mb) or the same special conditions are required to stimulate growth, both in cells and on bacteriologic media.

CHARACTER OF THE METABOLIC DEFICIENCIES

The special contribution of modern studies on the metabolism of factor-requiring and noncultivated species has been to strengthen the evidence that the critical problem is in the respiratory and probably not in the synthetic compartment.

Respiratory deficiencies.—In order to consider basic problems from the experimental point of view, Table 2 is constructed on the design used in Table 1. Evidence obtained from lung-grown tubercle bacilli indicates that pathogenic mycobacteria do not employ *in vitro*

[2] These observations alone should have warned me in 1941 that it was naive to hope that the tissue culture approach would yield ready success with *M. leprae*.

TABLE 2.—*Grouping of mycobacteria in relation to (A) ventilation of tissues, (B) respiratory equipment, and (C) production and use of growth catalysts.*

(A) Natural sites of infection	None	Pulmonary		Nonpulmonary		
		H-37Rv		*M. johnei*	*M. lepraemurium*	*M. leprae*
Mycobacteria	*M. phlei*	*in vitro*	*in vivo*			
(B) Cytochrome bands	++++	++	—	?	—	
Respiration/ subtrates	++++	++	—	v.sl.	—	
Optimal 0_2 for growth	20-40%	8-20%	?	4%	?	
(C) Mycobactin produced	++++	+	?	—	?	
utilized for growth	(+)[a]	(+)	?	+++[b]	—	

[a] Mycobactin requirements can be induced by factors which interfere with the synthesis of membranes.

[b] *M. johnei* and the wood pigeon bacilli, plus a large group of soil microbes, require microbially synthesized chelators of heavy metals. The latter are known to be important catalysts in respiration.

capacities for respiration while contending with the poor ventilation within infected tissues. Lung-grown tubercle bacilli resemble *M. lepraemurium.* They do not exhibit readily detectable cytochrome bands. Rates of respiration cannot be increased in the presence of nutritionally useful substrates.

For the transition from growth *in vivo* to growth *in vitro,* pathogenic species fall into at least three categories, as respects their cytochromes:

1. *Adaptive but complete cytochromes.*—This group includes tubercle bacilli and other species infecting ventilated tissues. They retain capacity to expand their cytochrome systems when removed from infected hosts. They also manufacture mycobactin, a growth catalyst, in extractable amounts.

2. *Catalyzable cytochromes.*—This group includes *M. johnei* and the wood pigeon bacilli. These presumably have but one weak site in their respiratory systems and seem analogous to one-step respiratory-deficient mutants. Mycobactin, a lipid-soluble chelator of heavy metals, is required to promote their cultivation on conventional media after removal from the host.

3. *Cytochromes with more than one weak site.*—This group includes *M. leprae* and *M. lepraemurium.* In their case the question arises if energy is acquired by some alternative system.

As a starting point for profitable investigation, the several unstudied and noncultivated species offer opportunity to ascertain which of these fall into categories 2 and 3 above and to determine if some may provide more instructive models than the factor-requiring strains now being investigated in our laboratory.

THE QUALITIES OF FACTOR-REQUIRING SPECIES

In another presentation Morrison[3] has explained why *M. johnei*, a factor-requiring species, is an interesting model for gaining insight into the more difficult problems to be anticipated in rat and human leprosy bacilli. Wheeler and I have broadened these studies by including the mycobacteria from Scandinavian wood pigeons, using both synthetic and complex media. From investigations conducted simultaneously *in vitro* and in cell cultures the following insights have been gained:

1. Neither Johne's bacilli nor the wood pigeon strains are fastidious in the sense of dependence upon complex or labile compounds produced by animal hosts. On the contrary, they grow on properly prepared synthetic media more readily than on complex nutrients. They fail to make the anticipated gain from exogenous mixtures of vitamins, amino acids, or peptides. These supplements, in fact, tend to be inhibitory, possibly because they induce unbalanced synthesis, i.e., synthesis at rates exceeding manufacture of the respiratory or mycobactin compartment. Given proper circumstances these organisms exhibit all the natural synthetic capacities of saprophytes and tubercle bacilli. It is extremely unlikely that they depend *in vivo* upon host enzymes or that they are favored by the biochemical environment provided by the internal milieu of animal cells.

2. These organisms, as Morrison has shown, can in fact readily be induced to grow without added mycobactin. For this purpose, their special needs are best accommodated by use of an undefined assortment of carbon fragments, prepared by autoclaving glucose and glycerol at pH 5.5, by the one-carbon fragment CO_2, and by a pH of 5.5, which apparently is required to facilitate transport of metals, organic acids and possibly other growth substrates.

3. Since accumulation of unoxidized acids, high CO_2, and low pH are characteristic of the phagocytic vacuoles rather than the "internal milieu" of cytoplasm within host cells, it may be surmised (a) that these species are not intracellular parasites, but obtain their relatively simple requirements from the outer surfaces of cell membranes which have folded into the tissue cell during phagocytosis, and (b) that within the phagocytic vacuoles of cells in nonpulmonary tissues, their growth probably is independent of mycobactin.

4. The results obtained by Wheeler in cell cultures of sheep and mouse monocytes add further support to the view that the conditions for growth on the surface of infolded cell membranes coincide with those required for independent growth *in vitro*. For example, if growth in cells exposed to normal atmosphere is poor, as with *M. johnei* strain 68, the rates are increased several-fold by the addition of mycobactin, i.e., by adding a factor that facilitates independent growth on conventional bacteriologic media.[3]

[3] It has since been found that ferric nitrate 1 μg/ml., when added to monocyte cultures in the presence of mycobactin, causes even more rapid intracellular growth.

The usefulness of CO_2, on the contrary, was first demonstrated to us when Wheeler employed a wood pigeon strain to explore the general significance of Chang's observation [1] that addition of CO_2 to normal atmospheres facilitates the growth of *M. lepraemurium* in cell cultures. In the same experiments CO_2 was found to enhance the growth of the wood pigeon strains on agar-containing synthetic medium at pH 5.5. Morrison and Rypka[4] have since shown the same to be true of *M. johnei* on synthetic media.

SUMMARY

Because of the foregoing observations, it is suggested that studies on the cultivation problem may be guided by the following principles:

1. In noncultivated species the basic problems are in the respiratory compartment. It is not known if these can be solved by supplementation of rudimentary cytochrome systems or if alternative systems must be devised.

2. Studies with the factor-requiring species do not suggest difficulties in the synthetic compartment. This makes it unlikely that complex or labile compounds from host cells will be required.

3. For growth studies, the physiologic conditions to be reproduced are those in the phagocytic vacuoles of cells residing in nonpulmonary tissues.

4. The effort to obtain growth in tissue cells in normal atmosphere presents problems analogous to those involved in direct growth *in vitro*. It appears that a significant observation in either system will be applicable to the other.

5. A number of "nonpulmonary" mycobacterial diseases are caused by noncultivated agents. One or more of these may yield organisms that prove to be more instructive models than the factor-requiring types now under study in our laboratory.

RESUMEN

Debido a observaciones anteriores, se sugiere que los estudios en el problema de cultivos, pueden ser guiado por los siguintes principios:

1. En especies no cultivadas el problema básico está en el compartimiento respiratorio. No se conoce si esto puede ser resuelto por suplementación de los sistemas citocrómicos rudimentarios o si deben desarrollarse sistemas alternativos.

2. Estudios con especies factor-requirentes no sugieren dificultades en el compartimiento sintético. Esto hace inprobable que se requieran los components complejos o lábiles de las células huéspedes.

3. Para los estudios de desarrollo, las condiciones fisiológicas a ser reproducidas son aquellas de las vacuolas fagocíticas de las células que residen en tejidos no pulmonares.

4. El esfuerzo para obtener crecimientos en células tisulares en atmósfera normal, presenta problemas análogos a aquellos envueltos en crecimientos directos *in vitro*. Parece que una observación significativa en cualquiera de los sistemas podrá ser aplicable al otro.

5. Un numero de enfermedades micobacterianas "no pulmonares" son causadas por agentes no cultivados. Uno o mas de estos puede ceder organismos que pueden probar

ser modelos más instructivos que los tipos factor-requirentes actualmente en estudio en nuestro laboratorio.

RESUMÉ

A la suite des observations rapportées, il est suggéré que les études ayant trait au problème posé par la culture des mycobactéries doivent obéir aux principes ci-dessous:

1. Dans les espèces non-cultivables les problèmes essentiels portent sur le compartiment respiratoire. On ignore se ces problèmes peuvent être résolus par l'apport supplémentaire d'un système cytochrome rudimentaire, ou s'il est necessaire de concevoir des systèmes alternatifs.

2. D'après les études menéces avec les espèces qui requièrent le facteur décrit, il ne semble pas exister de difficultés dans le domaine de la synthèse. Ceci fait qu'il est peu vraisemblable que des composés complexes ou labiles doivent être fournis par la cellule parasitée.

3. Pour les études sur la croissance, les conditions physiologiques qui doivent être reproduites sont celles des vacuoles physiologiques qui se trouvent dans les tissus non-pulmonaires.

4. L'effort requis pour obtenir une croissance dans des cellules tissulaires sous des conditions d'atmosphère normale présente des problèmes semblables à ceux rencontrés pour la croissance directe *in vitro*. Il apparait qu'une observation significative faite dans l'un des systèmes pourra être appliquée à l'autre.

5. Plusieurs maladies dues à des mycobactéries "non-pulmonaires" sont causées par des agents qu'il n'est pas possible de cultiver. Un ou plusieurs de ceux-ci peuvent libérer des organismes qui se révèleront être des modèles plus instructifs que les espèces qui requièrent le facteur dont il est question et qui sont actuellement à l'étude dans notre laboratoire.

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