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FACTORS THAT INFLUENCE THE GROWTH OF <u>MYCOBACTERIUM</u> LEPRAEMURIUM IN THE NAKAMURA'S SYSTEM

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One hundred years have been required to lay a basis for intelligent and rapid progress toward the in vitro cultivation of M. leprae. This encouraging situation has been brought about by the lucky coincidence of three constellations. Our own contribution has been the development of ultrasensitive determinations of ATP and demonstration that the results can be interpreted in terms of either growth potential or functional biomass. Two of the key contributions have been made by colleagues. Wilton Rightsel, Baptist Memorial Hospital, Memphis, Tennessee, (1,2) first published evidence that Mlm (Mycobacterium lepraemurium), when enclosed in diffusion chambers implanted in the peritoneal cavities of mice, was capable of slow growth. Our ATP data (3) confirmed that these growths were genuine and proved beyond doubt that Mlm is genetically capable of fabricating in vitro-type cell membranes under conditions that could be reproduced in vitro. Meanwhile, in 1972 and 1973, Masahiro Nakamura, Kurume Medical School, Japan, (4,5) published convincing evidence that Mlm could attain even higher growth rates in a system he had evolved while investigating the elongation Mlm cells, which occurred as a consequence of unbalanced The first slide shows the pattern that emerged while synthesis. confirming Nakamura.



F = Fetal calf serum. G = Goat serum.

44, 1 & 2

The 3-day ATP data demonstrate that the FBm (functional biomass = ATP per culture or aliquot) in Nakamura's semi-synthetic EK base fell to 50% of the original. Addition of the 7 supplements to complete the NC-5 medium reduced the physiologic sag by 50%, i.e., to 75% of the original. By 10 days the cells had recovered the energy levels utilized while growing in mice. By 20 days fetal calf serum was shown to be superior to the goat serum recommended by Nakamura. Thus, the purpose of the experiment had been accomplished in less than 3 weeks. More prolonged incubations demonstrated parallelisms between FBm and total biomass (number of cells x average length).

There are only two situations in which our experience does not coincide with that of Nakamura. In 1974 (6) he emphasized that small inoculums (0.1 x 10⁶ Bac / cult) grew more rapidly and more successfully than larger ones. This is contrary to the literature on associations between minimal successful inoculums and prolonged lag periods and contrary to our experience with cultivable bacteria. This slide (slide 2) shows that our experience with Mlm yielded classical results. The standard inoculum of 1 million cells/ ml (7 x10^b Bac/ Cult) was optimal. Inoculation of 10 or 100 times more cells did not shorten lag or increase growth rates. A ten-fold dilution of inoculum delayed the onset of growth only slightly. The inferior growth rate will be explained below. A 100-fold dilution of inoculum delayed the onset of growth for 5 or 6 weeks. The 1000-fold dilutions never produced demonstrable growth. The data suggest that 2-4 million cells per culture should provide a minimal standard inoculum.





a/ 7ml medium (24% air space) per culture.

The second point we could not confirm was the stability of the NC-5 medium. In our hands non-inoculated media deteriorated steadily in the dark at room temperature and at 30°. As a result, any factor that prolonged the lag period prevented cultures from attaining standard growth rates. In Fig. 2 the growth curve for 0.7 million cells per culture provides an example of this fact. The onset of growth from 0.7 million cells was only slightly retarded at 3 and 7 days. Nevertheless, the growth rate fell progressively behind the rates for larger inoculums.

Slide 2 reveals a phenomenon which never occurs with growth competent microbes. Note that the data are not expressed as absolute numbers but as % of original, which is equivalent to -fold increases. With cultivable microbes on conventional media the four successful inoculums would grow to essentially the same maximal population. Growth from the smallest successful inoculum of cells $(0.07 \times 10^6 \text{ Bac/cult})$ would proceed 10,000 times further than growth from the largest inoculum. With Mlm in NC-5 the four adequate populations peaked at the same time (6 weeks) and the populations differed by 1000 times. The period of successful growth and the extent of growth were controlled by time or by a fixed number of cell divisions and not by available oxygen or nutrition. Under the considerable variety of conditions investigated to date, termination of growth after 6 weeks has been a remarkably consistent phenomenon, one which we are now investigating with Dr. Nakamura.

Temperature. Mlm achieves its maximal rates of in vivo growth in the livers of mice at 38° . The Nakamura system is incubated at 30° . As seen in this slide (slide 3), incubation at 33° decreased the growth rate and increased elongation of the cells, a sign of unbalanced growth. Incubation at 36° aggravated these unfavorable effects of temperature. These findings apply to both in vivo grown and in vitro adapted cells, since incubating at 30° during their transition to in vitro type cells, then elevating temperature produced similar results. Growth at 30° and failure to grow at 38° reveals that Mlm remains non-cultivable at the body temperature of its natural hosts.

Elevation of incubation temperature is usually the easiest way to learn whether an organism has rate limitations in the ability to synthesize essential precursors, cofactors or metabolites; also whether a given medium is supplying the compounds required for maximal rates of growth. The significant facts are that even at 30° Mlm shows marked elongations; that NC-5 barely meets requirements for slow growth and that this organism and medium provide a challenging model of the physiologic ineptitudes which occur in so-called host dependent microbes.



Slide 3. Effect of incubation temperature on the expansion in FBm of <u>Mlm</u> in NC-5 medium.

6 ml medium (35% air space) per culture. Inoculum = 6 x 10^6 bacilli per culture.

<u>Merit of Compounds</u>. Slide 4 shows the results of quantitating the contribution made by each compound in the NC-5 medium. The data were obtained by deleting each compound separately. The fall-off in activity when a compound is absent defines its usefulness when present. For convenience the compounds have been grouped into three classes. Dr. Nakamura deserves great credit for the fact that every compound was useful at one stage or the other. When ranked (see insert) only α -ketoglutarate was of prime importance to both type 1 and type 11 cells. The usefulness of the remaining compounds fell into one pattern during stage 1 and a different pattern during stage 11.

In the period 1903 to 1972 thousands of cultivation trials by dozens of investigators had established <u>Mlm</u> as an "obligate intracellular parasite". Any system which initiates the growth of such an organism <u>in vitro</u> will be nonconventional in some readily recognized respect. Aside from key compounds the unique feature of the Nakamura system is that a cysteine-containing medium occupies 75% of the tube volumes. Displacement of air with a liquid medium is a convenient means of decreasing the amount of oxygen per culture and, hence, an empirical means of modifying ORP (oxidation-reduction potentials). The volumes of NC-5 medium were varied from 1 ml to 8.5 ml in culture tubes having a capacity of 9.2 ml.



Slide 4 Merits of different compounds in the NC-5 medium according to their usefulness for the growth of <u>Him</u>. 6 ml medium (35% air space) per culture. Inoculum = 6 x 10⁶ bac/culture.

Type 1 cells or cell membranes = those grown in vivo. Type 11 cells or membranes = in vitro-type, much more growth competent. Type 1 and 11 = the corresponding periods in the transition from host dependence to independence.

In order that we may appreciate the remarkable differences between MIm and the usual growth competent aerobes, this slide (slide 5) shows the growth response of Mycobacterium phlei under the foregoing experi-





44.1&2

mental conditions. As one would expect, irrespective of the test medium, a rapidly growing mycobacterium prefers all the oxygen it can obtain from normal atmospheres, i.e., 90% of the culture vessel occupied by air.

Now see what happens if one puts <u>Mim</u> instead of <u>M. phlei</u> in NC-5 medium under the same conditions (slide 6). Air spaces of 13% to 35%, i.e., 8.0, 7.0 and 6.0 ml medium permitted minimal physiologic "sag" of inoculated cells at 3 days. Greater air spaces (45 to 90% of tube volume) progressively and severely limited the ability of the cells to initiage growth.



Further incubation of the in vitro-adapted cells clearly established two points: (i) that oxygen must be restricted to obtain the maximal observed rates of growth and (ii) when oxygen becomes depleted the cells are severely damaged. (see the 8 and 8.5ml curves at 2, 4 and 6 weeks).

At 6 weeks the maximum growth occurred in cultures with 24% air space. At 8 weeks the maximum growth was in cultures having 35% air space. The maximum expansion of functional biomass occurred in cultures with 24% - 35% air space. Thus, ORP is a fundamental parameter, one which must be regulated for the sake of both in vivo-



grown and in vitro-adapted cells of Mlm.

At this point it became useful to learn whether cysteine was required simply as a reducing compound or also to furnish sulfhydryl groups. It, therefore, was replaced by various reducing substances in equimolar concentrations (slide 7). Sulfhydryl compounds such as glutathione and sodium thioglycolate were equivalent to cysteine, while the oxidized form of these compounds as well as other organic or inorganic reducing compounds were slightly inhibitory to the growth of <u>Mlm</u>. In the absence of any reducing substance in the medium the cells failed to expand the functional biomass.

Interestingly in the previous slide the three compounds that supported the growth of <u>Mlm</u> provided a carboxyl group in addition to the sulfhydryl group. When these compounds were replaced by sulfhydryls lacking a carboxyl group growth was not stimulated but inhibited (slide 8). Thus it seems that carboxylated sulfhydryls play a dual

24



role, that of reducing agent and also a donor of reduced sulfur for incorporation into enzymes, proteins, etc.

The elegant potentiometric systems, which employ ORP probes and plot results in millivolts, could not be adapted to our needs, because of the excessive size of the probes and also because metal electrodes become sulfide-fouled in systems poised by means of sulfhydryls. ORP dyes have been widely used to grade the quality of milk, to determine the rates of enzyme reactions, etc. but not as a routine monitor of ORP during the growth of fastidious microorganisms. By good fortune, it was known from other experience that negatively charged dyes do not damage cell membranes. We next demonstrated that indophenols have a negative charge and that <u>Mlm</u> will grow in the presence of useful concentrations of 2,6-dichlorophenolindophenol.

This slide (slide 9) shows two interesting points. 1. In the presence of 4 mg% of this dye <u>Mlm</u> grows at 97% of the usual rate. 2. Small inoculums of <u>Mlm</u> used did not modify the ORP of the NC-5 medium significantly during first four weeks of growth, after which the ORP of the cultures was essentially stable to the end of 12 weeks. The data at hand have failed to incriminate low ORP as the cause of growth cessation.





- Wiygul, W.C. and Rightsel, W.A. (1971). Growth of <u>M. lepraemurium</u> in diffusion chambers containing human embryonic skin cells and in cell-free chambers. J. Gen. Microbiol. <u>68</u>, 375-380.
- Dhople, A.M. and Hanks, J.H. (1973). Energetics of <u>M. lepraemurium</u> in diffusion chambers incubated in vitro and in mice. Infection and Immunity 8, 907-910.
- Nakamura, M. (1972). Multiplication of <u>M. lepraemurium</u> in cell-free medium containing α-ketoglutaric acid and cytochrome C. J. Gen. Microbiol. <u>73</u>, 193-195.
- Nakamura, M. (1973). Multiplication of <u>M. lepraemurium</u> in cell-free liquid medium. Proc. Japan Acad. <u>49</u>, 42-46.
- Nakamura, M. (1974). Quantitative multiplication of <u>M. lepraemurium</u> in a cell-free liquid medium. J. Gen. Microbiol. <u>82</u>, 385-391.

1976