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# PROGRESS IN THE RADIOLABELING OF MYCOBACTERIUM LEPRAE

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Attempts to utilize radioisotopes in studies of the metabolic characteristics of cultivable mycobacteria date back to at least 1953 (1). Attempts to adapt this methodology to Mycobacterium leprae, however, have not been made until relatively recently.

Three years ago I presented data to this group suggesting that viable leprosy bacilli could be identified in vitro by their ability to incorporate methyl-tritiated thymidine of high specific activity, and that this radiolabeling could be documented by autoradiography (2). Our data were published in the "Journal of Infectious Diseases" in 1972 (3). In January, 1974, Dr. Talwar's group in New Delhi published data in "Infection and Immunity" corroborating our work, and extending the technique to include electron microscopy autoradiography (4). The latter studies indicated that radioactivity was intimately associated with the leprosy bacilli. In June, 1974, Drs. Ambrose, Antia, and Khonolkar in Bombay reported the labeling of M. leprae with tritiated DOPA as well as tritiated thymidine. This work was published in "Nature" (5).

The purpose of my presentation today is to discuss the current status of studies pertaining to the radiolabeling of M. leprae, and to suggest methods for their improvement. I shall begin by briefly reviewing published data from my laboratory.

We have shown previously that the blood of patients with untreated lepromatous leprosy contains $10^6$ leprosy bacilli at all times, and that the majority of these bacilli are situated within monocytes. Two to 3% of the monocytes circulating in the blood of an untreated lepromatous patient harbor leprosy bacilli (6). Since the blood is infective for the footpads of mice, many of the bacilli within these cells are presumably viable (7).

When mononuclear phagocytes from the peripheral blood of bacteremic lepromatous patients are placed in tissue culture, they retain their M. leprae. Thus the blood is a convenient source for macrophages already infected with viable M. leprae. This is a 6 day old macrophage derived from the peripheral blood of such a patient.

If one exposes the tissue culture to a prolonged pulse of methyl-tritiated thymidine of high specific activity, and then prepares autoradiographs by the dipping emulsion technique, it can be seen that silver grains develop over the M. leprae. Presumably the bacilli have incorporated the radioisotope. Macrophages in tissue culture ordinarily fail to incorporate the tritiated thymidine because their nuclei are not synthesizing DNA. Thus there is an extremely low background in these experiments. Radiolabeling is successful only if tritiated thymidine of high specific activity is employed (3).

In unpublished studies we have examined M. leprae released into the tissue culture supernatant from dying macrophages on the grounds that M. leprae might prefer an extracellular environment. This slide shows that the bacilli have become greatly elongated after 2 weeks at 37° C. Such elongation is not novel, and has been noted in other systems by other investigators. However, some of the bacteria appear to be radiolabeled, suggesting that viable M. leprae are also present in the supernatant.

Although the slides shown could be superficially impressive, they must be interpreted carefully for 2 reasons:

a) We have never seen groups of grains over individual M. leprae; groups are seen only over groups of M. leprae in globi.

b) No more than 1-2% of grouped M. leprae on a given slide are unequivocally labeled, as we have stated in our published data.

Unfortunately it is impossible to judge the proportion of M. leprae radiolabeled with tritiated thymidine by Drs. Talwar (4) and Ambrose (5) since neither provides a denominator for his observations.

If the proportion of radiolabeled M. leprae is as low as our studies suggest, there are several possible reasons which must be considered:

1) The most obvious conclusion is that only a small proportion of bacilli are synthesizing DNA. This postulate is reasonable on 2 bases: (a) Only 1-10% of M. leprae in untreated lepromatous patients stain solidly. If this morphologic criterion of viability is correct, one would expect no more than 1-10% radiolabeling. The critical test of this thesis is to demonstrate that labeled bacilli are also solidly stained. We have not done this. (b) Human macrophages seldom persist in tissue culture more than 30 days, and M. leprae do not apparently multiply in such cultures. Therefore there is no opportunity to observe multiplication of those clones of M. leprae which might be synthesizing DNA.

2) Another possible conclusion is that radiolabeling might be artifactual. Either the microorganisms were not M. leprae or the grains did not reflect a radioactive event. The strictest possible controls are required to eliminate these possibilities. In our studies, there were no cultivable mycobacteria detected in the system. Further, there was no evidence of a chemical reaction between the photographic emulsion and carbol fuchsin or giemsa-stained M. leprae in terms of "positive chemography" (which refers to grain development secondary to a chemical rather than radioactive stimulus) or "negative chemography" (which refers to erasure of potential grains by chemical interference). No controls are noted in other published studies concerning tritiated thymidine uptake by M. leprae, and this factor greatly hinders the interpretation of those studies.

3) A third possibility is that tritiated thymidine is not an optimal radiolabel for M. leprae. This point deserves an explanation in terms of why we and, presumably, other investigators have preferred its use. First of all, we wished to identify small numbers of replicating leprosy bacilli within large numbers of phagocytic cells on morphologic grounds. This necessitated the use of autoradiography. Tritiated compounds are ideal for autoradiography because of their short path length in photographic emulsions. Silver grains can generally be identified directly over their point source of radioactivity. Tritiated thymidine itself has been a valuable tool for studying bacterial DNA

synthesis because of its incorporation into newly-synthesized DNA via the so-called "thymidine salvage pathway" utilizing thymidine kinase. We based the assumption that M. leprae would incorporate tritiated thymidine on the fact that Pasquier reported in 1963 that M. bovis strain BCG could be labeled in cell-free medium with tritiated thymidine (8). This required a tacit assumption that thymidine kinase was present in mycobacteria. There are several problems with this assumption:

(a) not all mycobacteria incorporate tritiated thymidine. Ghys reported in 1969 that M. smegmatis strain 607 would not incorporate methyl-tritiated thymidine in vitro using liquid scintillation methods (9). No autoradiographs were prepared. We, however, have obtained radiolabeling of wild-type M. fortuitum and M. smegmatis, leading to the possibility of strain variations.

(b) not all microorganisms possess thymidine kinase (10). Microorganisms devoid of this enzyme include Neurospora crassa, Saccharomyces cerevisiae, and Neisseria meningitis (11). What labeling is seen after these microorganisms are exposed to thymidine or thymidine nucleotides may be more closely related to RNA due to conversion of thymidine to uridine.

(c) we and others have made no attempt to demonstrate whether the label is within DNA. This is also true of Dr. Talwar despite the provocative title of his article which states that the thymidine is incorporated into M. leprae DNA (4).

Are there any other radioisotopes which should be considered of particula potential value in the radiolabeling of M. leprae? I shall confine my answer to systems with the potential for employing autoradiography since this is the area with which we are most familiar.

1) $^{3}$H-leucine: Dr. Dexter Howard at UCLA (12) and Dr. Richard Graybill (13) in my laboratory have shown that viable yeast-phase Histoplasma capsulatum can be identified within cultures of mouse and human macrophages, respectively, by the use of $^{3}$H-leucine to document protein synthesis. A mixture of tritiated amino acids could also be used. The problem with this approach is that macrophage protein synthesis must be blocked selectively by means of puromycin or a similar agent. This introduces an unwelcome variable when dealing with M. leprae, but the technology is straightforward.

2) $^{3}$H-adenine: Mr. Timothy Nealon in my laboratory has demonstrated the presence of viable Neisseria gonorrhoeae within cultures of human macrophages using $^{3}$H-adenine pulse labeling (14). The macrophage nuclei incorporate the label, but the bacteria are in the cytoplasm, and there is no resultant confusion as to location of silver grains.

3) $^{3}$H-uracil: Tritiated uracil is a promising radioisotope for M. leprae labeling, based on the successful tagging of the RNA of M. bovis strain BCG, M. kansasii, and M. intracellulare in cell-free cultures (15). Further, $^{14}$C-uridine is incorporated by M. tuberculosis (16). None of the published labeling studies has involved autoradiography.

An interesting study published by Ozato and Oiwa in "Infection and Immunity" in 1972 pointed out that mouse peritoneal macrophages showed retarded uptake of $^{3}$H-uridine several hours after being infected with M. lepraemurium (17). This study did employ autoradiography. In the photomicrographs provided, no labeled M. lepraemurium were shown although large numbers of microorganisms were present. The authors made no mention of whether mycobacterial labeling was sought. Drs. Rightsel and Sawyers have performed similar studies employing both M. lepraemurium and M. leprae, but have not employed autoradiography (18). No data were provided relative to bacterial labeling.

4) $^{3}$H-DOPA: Dr. Prabhakaran has studied for some years the possibility that leprosy bacilli possess a unique o-diphenoloxidase enzyme which oxidizes 3,4-dihydroxyphenylalanine (DOPA) to quinones which are then used as an energy source (19). The paper by Dr. Ambrose which has already been cited reports that $^{3}$H-DOPA will label M. leprae in a cell-free system employing drops of suspended M. leprae from human biopsy material in agar (5). Although a very provocative autoradiograph was provided which showed several apparently labeled bacteria, absolutely no details of the technique were given, and there was no mention of controls. Of great interest was the fact that granular bacilli were reported not to be labeled. This work is of very great potential importance, and requires full corroboration in other laboratories.

The future for successful radiolabeling of M. leprae in vitro with diverse compounds should be very bright. Autoradiography promises to be a powerful tool in this regard as it permits studies with small numbers of bacteria within their host cells, and allows an assessment of the metabolic activity of individual microorganisms. Tritiated compounds are especially likely to be useful in view of the short path length of tritium in photographic emulsions. This allows sharp localization of

the point-source of radioactivity. I have mentioned only a limited number of promising radiolabeled precursors; others may prove to be more useful. It will be necessary to use radiolabeled compounds of high specific activity to ensure that even low-level metabolic events will be documented. In studies of M. leprae within host cells, techniques will have to be developed to minimize the contribution to background of mammalian cell metabolic activity. The use of puromycin in experiments involving $^{3}$H-leucine labeling of H. capsulatum may not work with M. leprae. Experiments should be conducted to identify radiolabel uptake by extracellular M. leprae. Although these studies will necessarily be limited in duration as the bacilli die, they may provide a clue as to promising radiolabeled precursors which can be pursued in tissue cultures, in implanted diffusion chambers, or in the mouse footpad. High priority should be given to experiments which provide correlations between "solid staining" and radioisotope uptake, as the Bombay group has attempted to do. The need for exhaustive controls cannot be overemphasized. Cultivable mycobacterial contaminants have long been the bane of leprosy research. Autoradiography may pose another pitfall. This technique is simple in concept, but complicated in execution. Many traps await the unsuspecting investigator, which can be avoided only by the careful planning of control experiments.

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REFERENCES

1. Chiamori NY, Smith CR, Niashamara H, et al. Effect of Streptomycin on Assimilation of $^{14}$C from Acetate or Glucose by Human Tubercle Bacillus. Trans. Nat. Tuberc. Ass. Amer. pg 214, 1953.

2. Drutz DJ and Cline MJ. Tritiated Thymidine Labelling of M. leprae in the Human Lepromatous Macrophage. Int. J. Leprosy (abstract) 40:89, 1972.

3. Drutz DJ and Cline MJ. Incorporation of Tritiated Thymidine by Leprosy Bacilli in Cultures of Human Lepromatous Macrophages. J. Inf. Dis. 125:416, 1972.

4. Talwar GP, Krishnan AD, and Gupta PD. Quantitative Evaluation of the Progress of Intracellular Infection in vitro: Incorporation of $^{3}$H-Thymidine into Deoxyribonucleic Acid by Mycobacterium leprae in Cultivated Blood Monocytes. Infec. Immun. 9:187, 1974.

5. Ambrose EJ, Antia NH, and Khonolkar SR. Uptake of Radioactive DOPA by M. leprae. Nature 249:854, 1974.

6. Drutz DJ, Chen TSN, and Lu W-H. The Continuous Bacteremia of Lepromatous Leprosy. New Engl. J. Med. 287:159, 1972.

7. Drutz DJ, O'Neill SM, and Levy L. Viability of Blood-Borne Mycobacterium leprae. J. Inf. Dis. 130:288, 1974.

8. Pasquier JF. Utilization of Labeled Carbon ($^{14}C$) in the Study of Mycobacteria and Experimental Tuberculosis in Pasquier JF, Trnka L, and Urbancik R (eds), The Use of Radioactive Isotopes in Tuberculosis Research. Permagon Press, New York, 1965, pg 43.

9. Ghys R, Dernuet S, and Mankiewicz E. The Uptake of Radioactive Tracers by Saprophytic Mycobacteria. Amer. Rev. Resp. Dis. 100: 88, 1969.

10. Grivell AR and Jackson JF. Thymidine Kinase: Evidence for its Absence from Neurospora crassa and Some Other Micro-organisms, and the Relevance of This to the Specific Labelling of Deoxyribonucleic Acid. J. Gen. Microbiol. 54:307, 1968.

11. Jyssum S. Utilization of Thymine, Thymidine, and TMP by Neisseria meningitidis. 2. Lack of Enzymes for Specific Incorporation of Exogenous Thymine, Thymidine, and TMP into DNA. Acta Path. Microbiol. Scand. (B) 79:778, 1971.

12. Howard DH and Otto V. Protein Synthesis by Phagocytized Yeast Cells of Histoplasma capsulatum. Sabouraudia 7 :186, 1969.

13. Unpublished Data

14. Unpublished Data

15. Benitez P, Medoff G, and Kobayashi G. Rapid Radiometric Method of Testing Susceptibility of Mycobacteria and Slow-Growing Fungi to Antimicrobial Agents. Antimicrob. Ag. Chemother. 6:29, 1974.

16. Dickinson JM, Jackett PS, and Mitchison DA. The Effect of Pulsed Exposures to Rifampin on the Uptake of Uridine-$^{14}C$ by Mycobacterium tuberculosis. Amer. Rev. Resp. Dis. 105:519, 1972.

17. Ozato K and Oiwa K. Inhibition of $^{3}H$-Uridine Uptake in Mouse Macrophages Infected with Mycobacterium lepraemurium. Infec. Immun. 5: 255, 1972.

18. Rightsel WA and Sawyers MF. The Synthetic Capabilities of Mouse Macrophages Following in vitro Infection with Mycobacterium leprae and Mycobacterium lepraemurium. Int. J. Leprosy (abstract) 42:379, 1974.

19. Prabhakaran K. DOPA Metabolism by Mycobacterium leprae: Its Implications in Culture of the Bacillus and Chemotherapy of Leprosy. Lepr. Rev. 44:112, 1973.