Alternate Radiolabeled Markers for Detecting Metabolic Activity of Mycobacterium leprae Residing in Murine Macrophages

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This study demonstrated the utility of using 4% NaOH as a murine macrophage cell-solubilizing agent to discriminate between host macrophage metabolism and that of intracellular Mycobacterium leprae. A 4% concentration of NaOH had no deleterious effect on labeled mycobacteria. Thereby, alternate radiolabeled indicators of the metabolic activity of intracellular M. leprae could be experiment with. Significant incorporation of 14C-amino acid mixture, [14C]leucine, [14C]uridine, and carrier-free 32P was observed in cultures containing freshly extracted ("live") strains of M. leprae as compared with control cultures containing autoclaved bacilli.

One of the critical problems in Hansen’s disease has been the assessment of leprosy bacilli viability in a lesion. The more commonly used methods include a morphological index and replication of the bacilli in the mouse footpad. Recently, studies conducted by Mittal et al. (8) demonstrated the use of microculture techniques to monitor the metabolic activity of intracellular Mycobacterium leprae with [3H]thymidine. The present study was extended to identify alternate radiolabeled metabolic markers (such as 14C-amino acids, [14C]uridine, 32P, etc.) for M. leprae residing in murine macrophages. Since alternate labels would be incorporated into both intracellular mycobacteria and host cells, initial experiments were designed to develop techniques to discriminate between the metabolic activity of intracellular mycobacteria and that of host cells. These experiments were carried out with tritium-labeled M. fortuitum and 32P-labeled murine macrophages.

M. fortuitum ATCC 6841 maintained in chemically defined medium (2) was labeled with [3H]tyrosine (53.1 Ci/mmol; New England Nuclear Corp., Boston, Mass. [5 μCi/ml]). The labeled mycobacteria were harvested by centrifugation (10,000 × g for 20 min), washed, autoclaved, and suspended in RPMI 1640 medium (GIBCO Diagnostic; Madison, Wis.) enriched with 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO). Bacilli (4 × 107; equivalent to 8 × 105 dpm) were used for infecting the monocyte macrophage cultures maintained in flat-bottomed, 96-well microtiter plates (Costar, Cambridge, Mass.). The monocytes were derived from the peritoneal cavities of BALB/c mice by standard techniques (1). RPMI 1640 medium (200 μl; enriched with 20% [vol/vol] fetal calf serum) containing 1.25 × 105 peritoneal cells was distributed to each well and incubated at 37°C in a 5% CO2 incubator. After 24 h, the adherent growing monocytes were incubated with medium containing carrier-free 32P (285 Ci/mg, as H32PO4; ICN, Irvine, Calif. [3 μCi per well]) for 72 h. Subsequently, the wells were washed with prewarmed RPMI 1640 medium and replaced with medium containing 3H-labeled M. fortuitum for phagocytosis. After overnight incubation at 37°C, the cultures were washed repeatedly to remove extracellular bacteria, and the adherent macrophages were processed for harvesting. The microcultures were divided into three groups. Group 1 was harvested as such without any treatment, the macrophages containing the intracellular mycobacteria were stripped by using a rubber policeman, and the resulting cell suspension was dissolved in 1 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) and processed for liquid scintillation counting. Group 2 was subjected to alkaline digestion at 37°C for 60 min with 4% (wt/vol) NaOH (200 μl per well). The alkali-resistant, labeled mycobacteria in suspension were recovered by vacuum filtration.

Vacuum filtration was carried out with a vacuum manifold (VFM; Amicon Corp., Danvers, Mass.), and the mycobacteria were trapped with 0.45-μm (pore size) membrane filters (Nuclepore Corp., Pleasanton, Calif.). Group 3 was maximally treated, at first with ice-cold Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 0°C (50 μl per well; 0.2% [vol/vol] in phosphate-buffered saline [pH 7.2]), followed by trypsin (type II; Sigma) treatment for 45 min at 37°C (50 μl per well; 0.5% [vol/vol] in phosphate-buffered saline), and finally with NaOH (100 μl per well; 8% [vol/vol]) for 1 h at 37°C. The mycobacterial suspensions were filtered under vacuum to trap the mycobacteria on 0.45-μm (pore size) Nuclepore membrane filters. The Nuclepore membrane filters were dissolved in 1 ml of NCS at room temperature and processed for liquid scintillation counting. The disintegrations per minute of each isotope (3H and 32P) were corrected for background quenching, separated, and expressed independently of each other (10). Phagocytosis of the bacilli was confirmed by examining stained cover slips. By means of an inverted microscope (American Optical Corp., Buffalo, N.Y.), the stripping and solubilization of macrophages in the microtiter plate wells was constantly monitored. The efficacy of treatment of cultures leading to a reduction of 32P counts was determined by comparing the 32P disintegrations per minute observed in group 1 with the residual disintegrations per minute in groups 2 and 3 and expressed as a percentage. The percent efficacy of treatment was calculated as follows:

\[
100 - \frac{[(32P)DPMs \text{ in treated group 2 or 3}]}{[(32P)DPMs \text{ in group 1}]} \times 100
\]

The results of the dual-label experiment are shown in Table 1. The data show that 4% NaOH treatment of group 2 and the maximal treatment (see above) of group 3 cultures were effective in reducing the 32P counts to 185 ± 14 and 211
TABLE 1. Efficacy of the harvesting procedure to recover intracellular $^3$H-labeled M. fortuitum resident in murine macrophages

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Treatment</th>
<th>$^{32}$P DPM</th>
<th>$^3$H DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. $^{32}$P</td>
<td>-</td>
<td>635.5 ± 102.1</td>
<td>405.8 ± 55.3</td>
</tr>
<tr>
<td>M. $^{32}$P +</td>
<td>+</td>
<td>185.0 ± 14.2</td>
<td>359.0 ± 13.0</td>
</tr>
<tr>
<td>M. $^{32}$P +</td>
<td>+</td>
<td>211.3 ± 8.9</td>
<td>503.8 ± 27.5</td>
</tr>
</tbody>
</table>

$^a$ Mean disintegrations per minute of 10 samples plus or minus the standard error of the mean.

$^b$ $^3$H-labeled mouse peritoneal macrophages maintained in microtiter plates.

$^c$ $^3$H-labeled M. fortuitum (ATCC no. 6841) (4 × 10$^5$) was added to each well.

$^d$ A volume of 4% NaOH (wt/vol) was added (200 μl) per well and incubated at 37°C for 1 h. Subsequently, the suspensions were filtered with 0.45-μm (pore size) membranes (Nuclepore Corp.).

$^e$ $^3$H-labeled M. fortuitum (ATCC no. 6841) (4 × 10$^5$) was added to each well.

$^f$ $^3$H-labeled M. fortuitum (ATCC no. 6841) (4 × 10$^5$) was added to each well.

$^g$ $^3$H-labeled M. fortuitum (ATCC no. 6841) (4 × 10$^5$) was added to each well.

$^h$ $^3$H-labeled M. fortuitum (ATCC no. 6841) (4 × 10$^5$) was added to each well.

$^i$ $^3$H-labeled M. fortuitum (ATCC no. 6841) (4 × 10$^5$) was added to each well.

$^j$ $^3$H-labeled M. fortuitum (ATCC no. 6841) (4 × 10$^5$) was added to each well.

$^{14}$C-labeled mouse peritoneal macrophages were incubated with $^3$H-labeled M. fortuitum and then transferred to 10% fetal calf serum at 37°C for 1 h. After incubation, the nonphagocytosed bacilli were removed by washing with saline. A battery of alternate metabolic markers were used, including $^14$C-amino acids, $^14$C-leucine, etc., etc., in murine macrophages.

In succeeding experiments designed to monitor the uptake of alternate radiolabeled metabolic markers (such as $^14$C-amino acids, $^14$C-leucine, etc.) by M. leprae resident in murine macrophages, 4% NaOH treatment was followed by incubation at 37°C for 60 min was used to solubilize murine macrophages. The liberated intracellular radiolabeled bacilli were separated from the suspension by filtration with 0.45-μm (pore size) membrane filters. The experimental procedure is detailed below.

Radio labeling of M. leprae residing in murine macrophages

Macrophages maintained in microtiter plates were infected with M. leprae derived from armadillo liver and human skin biopsy (Fig. 1). The leprosy bacilli were screened for contaminants and cultivable mycobacteria by standard microbiological techniques. Only bacilli free of contaminants were used in the study. Bacilli (5 × 10$^3$) were incubated in RPMI 1640 medium containing 10% fetal calf serum were fed to each well. After overnight incubation, the nonphagocytosed bacilli were removed by washing and replaced with fresh warm medium. A battery of alternate metabolic markers were used, including $^14$C-amino acids, $^14$C-leucine, etc., etc., in murine macrophages.

FIG 1. Flow diagram describing the procedure for monitoring the uptake of radiolabeled compounds by M. leprae residing in murine macrophages. $^a$ $^14$C-amino acids, $^3$P, etc., etc., 4% concentration (wt/vol); M. $^{32}$P, macrophages; FCS, fetal calf serum; NS, normal saline (0.85% [wt/vol]); NS $\times$ tween 80, normal saline containing Tween 80 (1:2000); and NCS, tissue solubilizer.
recover the bacilli released into the supernatants during the culture period. At the time of harvest, the supernatants from each well were pooled with previous supernatants from that well with a modified Mash III automated sample harvester (Microbiological Associates, Los Angeles, Calif.). The wells were washed three times with normal saline (0.85% wt/vol), and the washings were collected in the tubes containing the supernatants. The combined supernatants were then incubated with NaOH at a final concentration of 4%. A volume of 200 μl of 4% (wt/vol) NaOH was added to each well. Both plates and tubes were incubated at 37°C for 60 min. The alkali-digested material in the plates was then pooled with the fluid in the tubes. This pooled mixture was filtered under vacuum to trap the labeled mycobacteria on 0.45-μm pore size Nuclepore membrane filters as described earlier. The tubes and the wells were rinsed three times with normal saline containing Tween 80 (1:2,000; Sigma). This was done to recover any adherent mycobacteria that might have been present; these washings were then filtered as before. The Nuclepore membrane filters were dissolved in 1 ml of NCS and processed for liquid scintillation counting as described earlier. The uptake of the radiolabeled compounds was corrected for background quenching, and results were expressed as disintegrations per minute.

The disintegrations per minute values are given in Table 2. Statistical analysis of the data showed significant differences in the radioactivity observed in macrophages containing autoclaved M. leprae and live M. leprae when incubated with 14C-amino acid mixture, [14C]leucine, [14C]uridine, and carrier-free 32P. The relatively higher incorporation of carrier-free 32P (7.4-fold) may be due to a spurt in the synthesis of phospholipids. In cultivable mycobacteria, more efficient labeling with 32P has been reported as compared with other isotopes (4).

The ability of M. leprae to take up a variety of radiolabeled compounds, such as 6-phosphogluconate, [14C]glucose, [3H]hypoxanthine, etc., has been reported previously (7, 15). Enzymes related to the tricarboxylic acid cycle, Embden-Meyerhoff, and hexose monophosphate pathways have also been demonstrated previously (15, 16). Therefore, M. leprae must possess metabolic pathways comparable to those of other microorganisms. Since M. leprae is an obligate intracellular pathogen, specialized techniques are required to study its metabolism. The alternate markers used in the study indicate the existence of biosynthetic pathways related to the production of proteins, ribonucleic acids, and phospholipid molecules in M. leprae. The radiometric assay of Nath et al. (9) and Mittal et al. (8) was limited by the use of [3H]thymidine and macrophages as host cells (which behave as end cells in the system). With the use of NaOH as an effective eucaryotic cell solubilizer, studies can now be extended to include replicating host cells and a variety of radiolabeled precursors utilized both by M. leprae and the host cell. The use of sodium hydroxide as a eucaryotic cell solubilizer is a well-established procedure in protein and DNA estimation of eucaryotic cells (11). The innate resistance of mycobacteria in general (6) and M. leprae in particular to 4% NaOH treatment is well known. Dhople and Storrs (3) showed no depreciation of ATP levels in bacilli before and after 4% NaOH treatment. Similarly, alkali-treated bacilli were found to be viable by the mouse footpad assay (12-14). Sodium hydroxide treatment is a recommended procedure for obtaining M. leprae suspensions free of host tissue components (17).

The alternate radiolabeled markers would have an added advantage since [3H]thymidine, an indicator of DNA synthesis, is limited to replicating organisms or related pathways. The alternate radiolabeled markers would be incorporated into mycobacterial products which have quantitatively a larger turnover (such as proteins, lipids, and nucleic acids), thereby increasing the efficiency of the radiometric assay. The technique offers a novel method of screening a variety of host cells (such as muscle cells, nerve cells, etc.) to generate the ideal microenvironment that the bacilli may require. In addition, a variety of radiolabeled metabolic markers can be tested in the system. In combination, these two possibilities can be used to further optimize the radiometric microculture assay system for monitoring the metabolism of intracellular M. leprae.

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LITERATURE CITED


| TABLE 2. Uptake of labeled compounds by intracellular M. leprae residing in murine macrophages |
|-----------------------------------------------|---|---|---|
| Radiolabeled compound | Autoclaved | Live | p* |
| 14C-amino acid mixture | 65.9 ± 15.6 (18) | 3,183.9 ± 166.2 (20) | < 0.001 |
| [14C]leucine | 1,898.0 ± 157.7 (10) | 2,527.3 ± 103.1 (8) | < 0.01 |
| [14C]uridine | 76.1 ± 3.4 (19) | 430.6 ± 44.6 (30) | < 0.001 |
| 32P | 8,171.4 ± 1,512.9 (10) | 60,443.0 ± 6,807.2 (10) | < 0.001 |

a M. leprae derived from human skin biopsy (no. 2743; experiments no. 1 and 3) and armadillo liver (no. 350; experiments 2 and 4) were free of contaminants as checked on blood agar and Lowenstein-Jensen media. Acid-fast bacilli (5 × 106) were fed to each well containing 48-h-old mouse macrophages.

b Isotopes were added after phagocytosis of the mycobacteria as follows: 1 μCi of 14C-amino acid mixture (50 mCi/mmol), [14C]uridine (52.4 mCi/mmol), and [14C]leucine (300 mCi/mmol) and 3 μCi of carrier-free 32P as H35PO4 (285 Ci/mg), respectively, per well.

c Freshly extracted M. leprae.

d Compared with autoclaved M. leprae (Student’s t test). One tailed.

e Number of samples.


