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Use of Mono Mac 6 Human Monocytic Cell Line and J774 Murine Macrophage Cell Line in Parallel Antimycobacterial Drug Studies

E. L. WRIGHT,[1] D. C. QUENELLE,[2] W. J. SULING,[3] AND W. W. BARROW[1]*

Mycobacteriology Research Unit,[1] Infectious Disease Group,[2] and Bacteriology and Mycology Research Unit,[3] Southern Research Institute, Birmingham, Alabama 35205

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The Mono Mac 6 (MM6) human monocytic cell line was evaluated with the established J774 murine macrophage cell line to ascertain its effectiveness in determining the intracellular activities of antimycobacterial drugs. Cells were infected with *Mycobacterium tuberculosis* H37Ra and treated with drug concentrations corresponding to the MICs, as well as to threefold higher than and threefold less than the MICs. Changes in CFU were compared after 7 days to determine significant differences between treated and nontreated groups. The results suggest that MM6 will make a useful model for testing the intracellular activities of antituberculosis drugs.

With the increasing prevalence of mycobacterial infections, the development of new antimycobacterial agents and strategies for treating mycobacterial infections is of paramount importance. While in vitro methods such as radiometric and colorimetric assays are important to determine the MICs of antimicrobics, effective doses of antimycobacterial drugs should also be evaluated in a macrophage model to ensure intracellular drug effectiveness.

In this regard, several macrophage models have been used. A variety of murine macrophage models have been described; however, the one that appears to be most commonly used is the J774 macrophage cell line (7, 9, 13). For studies involving human cells, peripheral blood monocyte-derived human macrophages appear to be favored (6, 8). However, because there are no fully differentiated human macrophage cell lines, the only human monocytic cell lines available for parallel use with murine cell lines are those requiring stimulation.

Recently, a human monocytic cell line has been described that may be useful for antimycobacterial studies. The Mono Mac 6 (MM6) cell line appears to be the only human cell line to constitutively express phenotypic and functional features of mature monocytes (14). Unlike the U937 and THP-1 human monocytic cell lines that must be induced to develop phagocytic properties (4), the MM6 cell line has the ability to constitutively phagocytize antibody-coated erythrocytes (14) and mycobacteria (3, 10).

The primary goal of this investigation was to use the MM6 cell line in a comparative study with the J774 murine macrophage cell line to examine the intracellular effectiveness of antimycobacterial drugs. This makes it possible to examine the intracellular effectiveness of antimycobacterial drugs at two levels. The results obtained with the J774 cell line can be used along with parallel studies in infected mouse models, and the results obtained with the MM6 cell line can be used to help predict intracellular activity in humans. By using both models, it should be possible to obtain a broader range of therapeutic information for antimycobacterial agents.

The MM6 cell line was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. This cell line is a human acute monocytic leukemia cell line, which was originally established by H. W. L. Ziegler-Heitbrock, Institute of Immunology, University of Munich, Munich, Germany (14). The cells were maintained in RPMI 1640 containing 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, nonessential amino acids, 1 mM sodium pyruvic acid, and 9 μg of bovine insulin (Sigma Chemical Co., St. Louis, Mo.) per ml.

Prior to use in macrophage experiments, *Mycobacterium tuberculosis* H37Ra (ATCC 25177; American Type Culture Collection, Rockville, Md.) was initially grown in Middlebrook 7H9 (Difco Laboratories, Detroit, Mich.) containing 0.5% Tween 80 (Sigma) and 10% albumin-dextrose-catalase (ADC) (Difco). After reaching exponential phase, the mycobacteria were dispersed by vortexing with glass beads and were allowed to settle for 30 min (11). The supernatant was removed, frozen at −70°C, and then thawed and used for infection by resuspension in appropriate cell culture medium (11).

Before infection, MM6 cells were adjusted to 8×10^5 cells per ml, and 0.5 ml per well was dispensed in 12-well tissue culture dishes (Corning Costar Corp., Cambridge, Mass.). Mycobacteria were then added to the MM6 cells to achieve a final ratio of 20 mycobacteria per macrophage, with a density of 4×10^5 MM6 cells per 1.0 ml per well. After an infection interval of 4 h, infected MM6 cells were centrifuged ($200 \times g$) and washed twice with Dulbecco's phosphate-buffered saline (PBS; Sigma) to remove any free mycobacteria. The cells were plated at a density of 4×10^5 cells per 1.0 ml per well, with appropriate wells containing antimycobacterial drugs, and the wells were incubated at 37°C with 5% CO_2. At day 4, 1.0 ml of fresh medium was added to each well. To determine the number of CFU at zero hour, the cells were lysed with 0.25% (wt/vol) sodium dodecyl sulfate (SDS) in PBS, serially diluted, and plated onto 7H10 agar plates (1, 8). To decrease viscosity and facilitate serial dilutions, 5 μl (5 U of activity) of RQ DNase (Promega Corp., Madison, Wis.) with $MgSO_4$ (5 mM) was added to each well after the addition of SDS. The procedure was repeated at 7 days postinfection. CFU were enumerated after 2 to 3 weeks.

J774 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. J774 cells were plated at a concentration of 2×10^5 cells per ml per well in 12-well tissue culture dishes (Costar) and were allowed to adhere overnight. After 24 h, the

* Corresponding author. Mailing address: Mycobacteriology Research Unit, Southern Research Institute, 2000 Ninth Ave. South, Birmingham, AL 35205. Phone: (205) 581-2139. Fax: (205) 581-2877.

TABLE 1. CFU obtained from MM6 and J774 cells infected with *M. tuberculosis* H37Ra and treated with RIF, INH, and EMB[a]

Drug	Cell line	Dilution factor	CFU				
			Zero h	NT at 7 days[b]	MIC at 7 days[c]	MIC−3 at 7 days[c]	MIC+3 at 7 days[c]
RIF	MM6	10^4	8.7 ± 0.88	184 ± 28[d]	14.2 ± 1.8[d]	206 ± 38	0.89 ± 0.18[d]
	J774	10^4	84.8 ± 5.4	235 ± 19[d]	158 ± 24[e]	227 ± 23	11.8 ± 0.40[d]
INH	MM6	10^3	23.9 ± 5.7	226 ± 56[d]	0.45 ± 0.29[d]	235 ± 6.0	<0.1[d]
	J774	10^3	73.5 ± 9.3	372 ± 24[d]	26.7 ± 3.6[d]	359 ± 53	3.1 ± 0.81[d]
EMB	MM6	10^3	45.8 ± 6.4	304 ± 52[d]	2.4 ± 1.6[d]	15.4 ± 4.2[d]	1.78 ± 0.52[d]
	J774	10^3	132 ± 14	625 ± 84[d]	18.9 ± 4.1[d]	365 ± 70[e]	3.5 ± 0.8[d]

[a] Infected cells were treated with concentrations equivalent to a predetermined MIC, a MIC−3, and a MIC+3. The numbers of CFU were determined immediately after infection (zero hour) and at 7 days postinfection. The values given are the accumulative data from three separate experiments conducted in triplicate and are the means ± standard errors of the means (n = 18 for CFU determined at zero hour; n = 9 for all other groups).

[b] One-way analysis of variance was used to determine significant increases in the numbers of CFU for the nontreated cells (NT) from zero hour to 7 days.

[c] One-way analysis of variance was used to determine significant differences between the nontreated group at day 7 and each drug-treated group at day 7. A correction for multiple comparisons (posttest) was performed by means of the Tukey-Kramer multiple comparisons test.

[d] $P < 0.001$.

[e] $P < 0.01$.

medium was removed and replaced with medium containing 1% serum to reduce cell proliferation (9). At 48 h, adherent cells were enumerated by means of an ocular grid to calculate the infection ratio (5). *M. tuberculosis* H37Ra was then suspended in RPMI 1640 containing 1% fetal bovine serum, and the suspension was dispensed into individual wells at a density of 5 mycobacteria per macrophage (9, 13). The infected cells were then incubated at 37°C in an atmosphere of 5% carbon dioxide for 4 h. The supernatant was aspirated, and the cells were washed twice with Dulbecco's PBS (Sigma) to remove unphagocytosed mycobacteria. Fresh medium (1.0 ml), with or without antimycobacterial drugs, was then added to each well. The cells were maintained and the numbers of CFU were determined as described above. Cell lines were routinely assayed for mycoplasma contamination by means of the Gen-Probe *Mycoplasma* Rapid Detection system (Gen-Probe, San Diego, Calif.).

Cell viability was assayed by means of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [Thiazolyl Blue]) Cytotoxicity Assay kit ZA0022 (Advanced Tissue Sciences, Inc., La Jolla, Calif.). Assays were performed in triplicate and were averaged, and a coefficient of variance was calculated. Toxicity was determined by comparing the absorbance (measured in optical density units) of each treated set with the mean optical density values of the cell control cultures and was expressed as a percent of control (2). Percent viability values after 7 days at the highest drug concentrations were 102 ± 7.6 (isoniazid [INH]), 99.7 ± 5.6 (rifampin [RIF]), and 78.4 ± 11 (ethambutol [EMB]) for J774 cells and 94.3 ± 13 (INH), 94.1 ± 6.9 (RIF), and 86 ± 6.3 (EMB) for MM6 cells (n = 9).

The MIC of each drug was determined by a broth microdilution assay with Alamar Blue (Sensititre/Alamar, Westlake, Ohio) and 7H9 broth supplemented with 10% ADC enrichment and 0.2% glycerol (12). A MIC was considered the lowest drug concentration which produced a differential absorbance (A_{570} [reduced dye] − A_{600} [oxidized dye]) of ≤0.0 as measured with an optical microplate reader. The MICs against *M. tuberculosis* H37Ra were 4 to 8 μg/ml for EMB, 0.03 to 0.06 μg/ml for INH, and 0.002 μg/ml for RIF. In the studies performed here, the MICs used were the lowest in each range, i.e., 4 μg/ml for EMB, 0.03 μg/ml for INH, and 0.002 μg/ml for RIF. Three concentrations of each drug were used: the MIC, a concentration threefold less than the MIC (MIC−3), and a concentration threefold greater than the MIC (MIC+3). For all drugs tested in both cell lines, a significant decrease in the numbers of CFU ($P < 0.01$) was observed for concentrations equal to the MIC and the MIC+3. The results of three separate experiments, each of which was conducted in triplicate, for each drug and cell line are combined in Table 1 for statistical evaluation. With EMB, a significant decrease in the numbers of CFU was also observed at MIC−3 (Table 1).

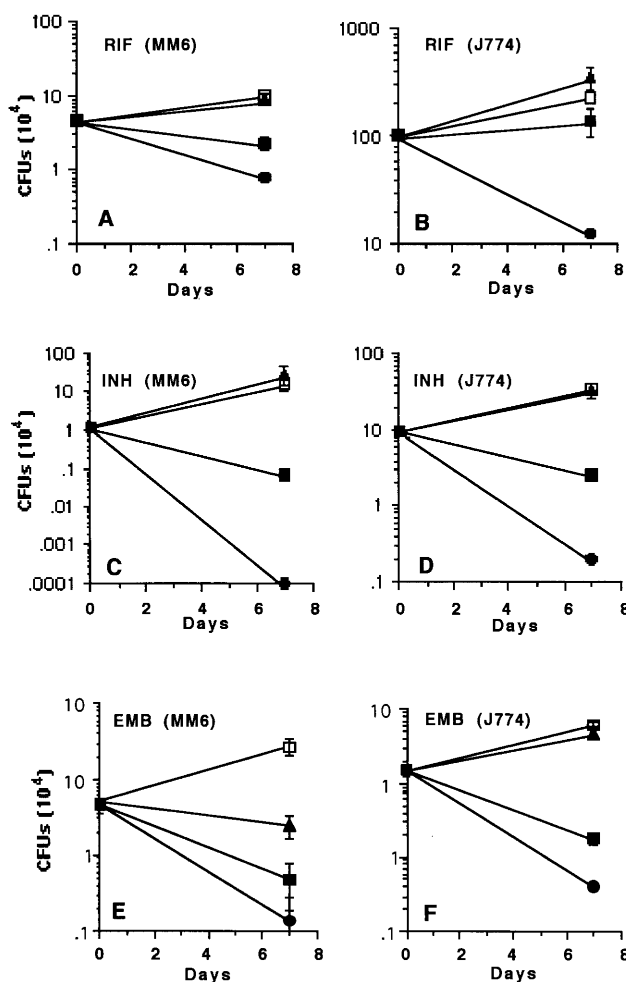

FIG. 1. Determination of numbers of CFU of *M. tuberculosis* H37Ra-infected monocytic cell lines at time zero and 7 days postinfection. The results reported are from one experiment conducted in triplicate and are means and standard errors of the means (bars). ■, MIC; ●, MIC+3; ▲, MIC−3; □, nontreated cells. Details are given in the text.

As seen in Table 1, the most obvious difference between MM6 and J774 macrophages is between their infection rates. It was necessary to infect the MM6 cell line with 20 mycobacteria per macrophage, while 5 mycobacteria per macrophage were sufficient for infection of J774 cells. In the set of experiments with RIF (Table 1 [zero hour]), the infection ratios were $8.7 \times 10^4 \pm 0.88 \times 10^4$ CFU per 4×10^5 monocytes for MM6 versus $84.8 \times 10^4 \pm 5.4 \times 10^4$ CFU per 4×10^5 monocytes for J774, the means being statistically different ($P < 0.0001$). In the set of experiments with INH (Table 1 [zero hour]), the infection ratios were $23.9 \times 10^3 \pm 5.7 \times 10^3$ cells per 4×10^5 monocytes for MM6 cells versus $73.5 \times 10^3 \pm 9.3 \times 10^3$ CFU per 4×10^5 monocytes for J774 ($P = 0.0005$), and in the set of experiments with EMB (Table 1 [zero hour]), the infection ratios were $45.8 \times 10^3 \pm 6.4 \times 10^3$ CFU per 4×10^5 monocytes for MM6 versus $132 \times 10^3 \pm 14 \times 10^3$ CFU per 4×10^5 monocytes for J774 cells ($P = 0.0002$). Statistical analyses were conducted by means of the alternate Welch *t* test (InStat 2.01; GraphPad Software, San Diego, Calif.). Both cell lines routinely demonstrated a significant increase in the numbers of CFU from time zero to the end of infection at 7 days for the nontreated cells.

The graphs in Fig. 1 show single representative experiments, each of which was conducted in triplicate. These graphs show that intracellular evaluation of RIF, INH, and EMB by both cell lines gives comparable findings. Although some parameters vary between the J774 and MM6 cell lines, the results indicate that the MM6 cell line is an effective and easily handled model that can be reliably used to evaluate the intracellular activities of antimycobacterial drugs.

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