ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1996, p. 1542–1544 Vol. 40, No. 6
0066-4804/96/$04.00+0

NOTES

Reporter Gene Technology To Assess Activity of Antimycobacterial Agents in Macrophages

TARAQ M. ARAIN,* ANNA E. RESCONI, DEVINDER C. SINGH, AND C. KENDALL STOVER

Department of Molecular Microbiology, PathoGenesis Corporation, Seattle, Washington

Received 27 October 1995/Returned for modification 7 February 1996/Accepted 2 April 1996

Reporter strains of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG endogenously expressing firefly luciferase were used in bioluminescence assays to evaluate the activities of isoniazid and rifampin against mycobacteria sequestered in human macrophages. This methodology allowed the efficacy of antibiotics against intracellular mycobacteria to be assessed without the labor-intensive procedures and protracted incubation requirements associated with conventional CFU determinations.

The remarkable propensity of members of the pathogenic mycobacteria to survive within the intracellular environment of the macrophage poses particular problems for an antimicrobial agent. To exert an effect, the antibiotic must be able to penetrate the mammalian cell membrane, remain stable within the hostile cellular environment, and then achieve appreciable concentrations at the intracellular site in which the pathogen resides. The burden of organisms sequestered within macrophages during the course of illness represents a sizable source of viable organisms, so that the ability to ascertain whether an antibiotic can affect these organisms should be a useful criterion to predict therapeutic efficacy. Most studies of this nature have employed direct measurements of CFU to determine numbers of viable bacteria within the eukaryotic cells after antibiotic treatment (3, 7, 8, 10). Principal drawbacks to these methods include the extra time which must be provided to allow countable colonies to appear on solid media and the possibility of contaminants appearing on the agar plates, which can confound final results. Strategies employing the firefly luciferase gene as a reporter of mycobacterial viability have been applied to the evaluation of antimicrobial agents both in vitro (1, 2, 5) and in vivo (4) and have largely overcome these obstacles. We investigated the feasibility of employing recombinant organisms expressing firefly luciferase to assess the activity of antimycobacterial compounds ex vivo in a human macrophage system.

Human THP-1 monocytic cells (ATCC TIB-202) were differentiated into macrophages by treatment with 50 ng of phorbol 12-myristate-13-acetate (Sigma Chemical Co., St. Louis, Mo.) and were distributed at a density of 4×10^5/ml to wells of a 48-well flat-bottom microtiter plate. After a 48-h incubation at 37°C in a 5% CO_2 atmosphere, cell monolayers were washed with RPMI 1640 medium (GIBCO BRL, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah) and were infected with a logarithmic-phase bacterial culture at a 1:1 multiplicity of infection. Bacterial aggregates were dispersed prior to infection by sonication for 20 s in a Vibracell cup horn sonicator (Sonics and Materials Inc., Danbury, Conn.) at a 20% amplitude with a 0.1-s on-off pulse cycle. The strains used were *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) and *M. bovis* BCG Connaught (ATCC 35745), both transformed with pMV361-*lux*. Details of the construction of this vector and identification of transformants have been described in detail previously (4). After incubation for 4 h at 37°C in a 5% CO_2 atmosphere, the cells were washed five times with Hanks balanced salt solution (GIBCO BRL) to remove bacteria in the extracellular milieu. Eukaryotic cells were lysed by addition of 0.2 ml of phosphate-buffered saline containing 1% Triton X-100. Aliquots of 0.1 ml of the lysate

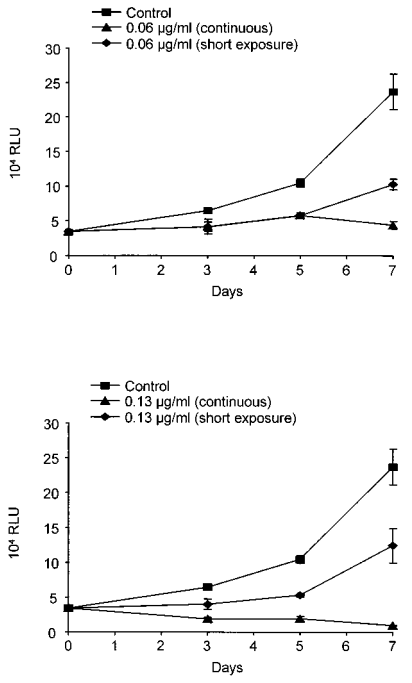

FIG. 1. Effects of isoniazid at two different concentrations on intracellular *M. bovis* BCG determined by assay of bioluminescence. The isoniazid was either left in contact with the macrophages throughout the course of the experiment (continuous exposure) or removed after 90 min by washing the cell monolayer (short exposure). Error bars, standard deviations from the means for duplicate measurements.

* Corresponding author. Mailing address: PathoGenesis Corporation, 201 Elliott Ave. West, Seattle, WA 98119. Phone: (206) 467-8100. Fax: (206) 282-5065. Electronic mail address: tarain@path.path.com.

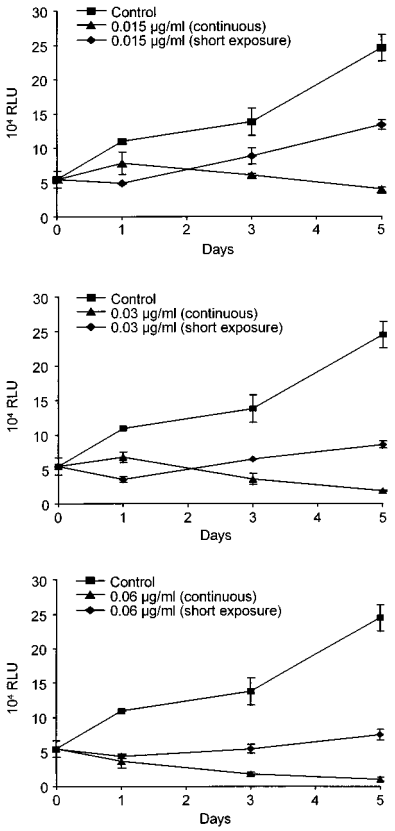

FIG. 2. Effects of rifampin at three different concentrations on intracellular *M. bovis* BCG determined by assay of bioluminescence. The rifampin was either left in contact with the macrophages throughout the course of the experiment (continuous exposure) or removed after 90 min by washing the cell monolayer (short exposure). Error bars, standard deviations from the means for duplicate measurements.

were transferred to an opaque 96-well microtiter plate (MicroLite 1, Dynatech Inc., Chantilly, Va.), and luminescence determinations were made in a Wallac Microlumat LB96P luminometer (Wallac Instruments, Gaithersburg, Md.) to obtain a day zero pretreatment reading. Alternatively, 0.2-ml aliquots were transferred to a Falcon 2054 test tube and relative light unit measurements were made with a Wallac Autolumat LB 953 instrument. The luminometer automatically injected 0.1 ml of 1 mM luciferin (R & D Systems, Minneapolis, Minn.) prepared in 0.1 M trisodium citrate (pH 5.1) into each tube or well. Luminescence was measured for 15 s and expressed in relative light units. Fresh medium (RPMI plus 10% fetal bovine serum) was added to the remaining wells. To investigate the effects of antimycobacterial drugs, the medium was supplemented with selected concentrations of isoniazid or rifampin (Sigma) prepared in sterile deionized water or dimethyl sulfoxide (Sigma), respectively. The drug was either left in contact with the cells throughout the course of the experiment or was removed after 1.5 h by washing the monolayer with fresh RPMI medium. Cells in duplicate wells were lysed at desired daily intervals, and the bioluminescence assay described above was performed.

Continuous exposure of BCG-infected cells to 0.06 and 0.13 μg/ml of isoniazid completely inhibited bacterial growth (Fig. 1). All three concentrations of rifampin tested proved inhibitory to intracellular proliferation of BCG (Fig. 2). Exposure of the infected cells to isoniazid or rifampin for only 90 min

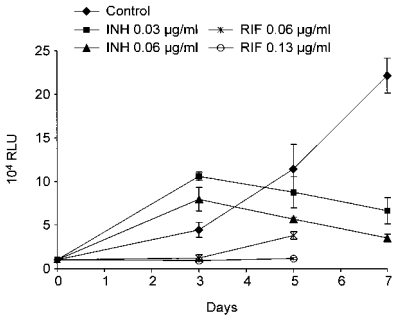

FIG. 3. Effects of isoniazid (INH) and rifampin (RIF) on intracellular *M. tuberculosis* determined by assay of bioluminescence. The drugs were left in contact with the macrophages throughout the course of the experiment. Error bars, standard deviations from the means for duplicate measurements.

retarded bacterial growth within the macrophages, although the effects were not as pronounced as those observed with continuous drug exposure. Both of these antimicrobial agents are known to accumulate rapidly within phagocytes (6, 9), and evidence exists to indicate that the uptake of rifampin occurs through passive mechanisms (9). It is therefore possible that short-term exposure of the macrophages either did not allow a sufficient amount of the drug to enter the eukaryotic cell or that the antibiotic diffused out of the macrophage after exposure during the washing step.

Both isoniazid and rifampin were shown by the bioluminescence assay to be highly active against intracellular *M. tuberculosis* at the concentrations tested (Fig. 3). Results strongly indicated that rifampin elicited a much more rapid antimycobacterial response than isoniazid. Rifampin inhibited the growth of intracellular bacteria at the same concentration as the MIC determined in vitro, in accord with the findings of Clini and Grassi (1a).

This application of luciferase reporter gene technology to assess the activity of antimicrobial agents against intracellular mycobacteria represents the final facet of a complete strategy employing bioluminescence-based assays for in vitro and in vivo drug evaluation (1, 4). The main attraction of the reporter gene system is the freedom from having to plate multiple dilutions of cell homogenates at the end of the experiment and then wait several weeks for colonies to appear. The bioluminescence assay can provide a direct indication of bacterial growth throughout the course of the experiment without these time-consuming steps. As such, this methodology should find relevance in all studies involving the interactions of mycobacteria and macrophages in which bacterial numbers must normally be determined by plating.

REFERENCES

1. **Arain, T. M., A. E. Resconi, M. J. Hickey, and C. K. Stover.** 1996. Bioluminescence screening in vitro (Bio-*Siv*) assays for high-volume antimycobacterial drug discovery. Antimicrob. Agents Chemother. **40:**1534–1539.

1a. **Clini, V., and C. Grassi.** 1970. The action of new antituberculous drugs on intracellular tubercle bacilli. Antibiot. Chemother. (Basel) **16:**20–26.

2. **Cooksey, R. C., J. T. Crawford, W. R. Jacobs, Jr., and T. M. Shinnick.** 1993. A rapid method for screening antimicrobial agents for activities against a strain of *Mycobacterium tuberculosis* expressing firefly luciferase. Antimicrob. Agents Chemother. **37:**1348–1352.

3. **Dhillon, J., and D. A. Mitchison.** 1992. Activity *in vitro* of rifabutin, FCE 22807, rifapentine, and rifampin against *Mycobacterium microti* and *M. tuberculosis* and their penetration into mouse peritoneal macrophages. Am. Rev. Respir. Dis. **145:**212–214.

4. **Hickey, M. J., T. M. Arain, R. M. Shawar, D. J. Humble, M. H. Langhorne, J. N. Morgenroth, and C. K. Stover.** 1996. Luciferase in vivo expression

technology: use of recombinant mycobacterial reporter strains to evaluate antimycobacterial activity in mice. Antimicrob. Agents Chemother. **40:**400–407.
5. **Jacobs, W. R., Jr., R. G. Barletta, R. Udani, J. Chan, G. Kalkut, G. Sosne, T. Kieser, G. J. Sarkis, G. F. Hatfull, and B. R. Bloom.** 1993. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. Science **260:**819–822.
6. **Johnson, J. D., W. L. Hand, J. B. Francis, N. Jing-Thompson, and R. W. Corwin.** 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med. **95:**429–439.
7. **Mor, N., and L. Heifets.** 1993. MICs and MBCs of clarithromycin against *Mycobacterium avium* within human macrophages. Antimicrob. Agents Chemother. **37:**111–114.
8. **Onyeji, C. O., C. H. Nightingale, D. P. Nicolau, and R. Quintiliani.** 1994. Efficacies of liposome-encapsulated clarithromycin and ofloxacin against *Mycobacterium avium-M. intracellulare* complex in human macrophages. Antimicrob. Agents Chemother. **38:**523–527.
9. **Prokesch, R. C., and W. L. Hand.** 1982. Antibiotic entry into human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **21:**373–380.
10. **Sbarbaro, J. A., M. D. Iseman, and A. J. Crowle.** 1992. The combined effect of rifampin and pyrazinamide within the human macrophage. Am. Rev. Respir. Dis. **146:**1448–1451.