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Rapid In Vitro Metabolic Screen for Antileprosy Compounds

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Measurement of intracellular ATP of *Mycobacterium leprae* after direct in vitro exposure to antimicrobial agents was evaluated as a rapid means of identifying potentially useful therapeutic agents. Nude mouse-derived *M. leprae* was incubated in an axenic modified Dubos medium in the presence or absence of antimicrobial agents for up to 3 weeks. ATP was then assayed by using the firefly bioluminescence technique. Rifampin, clofazimine, and ethionamide each effected a significantly accelerated rate of ATP decay compared with controls. Dapsone appeared inactive, possibly reflecting a general insensitivity of this system to compounds acting at certain loci. The system appeared suitable for assessing comparative activity of new structural analogs of clofazimine. Other active compounds included erythromycin, minocycline, chloramphenicol, gramicidin, and, to a lesser extent, cycloserine, cephalothin, ciprofloxacin, tetracycline, and gramicidin S. The penicillins, bacitracin, isoniazid, nalidixic acid, trimethoprim, polymyxin B, and griseofulvin were all inactive. The system appears sensitive to agents with various modes of action and may prove useful as a primary screen for antileprosy drugs.

Mycobacterium leprae, the etiologic agent of leprosy, has yet to be cultivated in vitro. Therefore, primary screening for new drugs as well as susceptibility testing of clinical isolates is conducted by using a mouse footpad technique (17). This method is expensive and cumbersome and requires up to a year or more to complete. In addition, the first screening of a new compound usually requires 10 to 15 g of drug (21). In vitro drug assays, involving uptake of radiolabeled macromolecular precursors by *M. leprae* residing within macrophages, have been described (15) but are still relatively expensive and cumbersome for a primary drug screen. Measurement of intracellular ATP has been used as an index of the metabolic status of *M. leprae* isolated from armadillos (1–3, 6, 7, 13) and from human biopsies after drug treatment (4); it has also been used to evaluate the potential of in vitro culture media (5, 14).

A close correlation between ATP levels and viability in the mouse footpad has been noted in a number of these studies (3–5). The present paper evaluates the potential of an in vitro incubation system and ATP analysis as a rapid primary screen for identifying compounds with antileprosy activity.

MATERIALS AND METHODS

Inoculum preparation. Footpads of athymic nude mice, infected 6 to 12 months previously with 5×10^7 *M. leprae*, were surface decontaminated by 1 to 2 min of UV irradiation, 2 min in ethyl ether, and 10 min in Acudyne (Acme United Corp., Bridgeport, Conn.), followed by rinsing in 70% ethanol. Footpads were then minced with scissors and homogenized with a Ten Broeck homogenizer (Wheaton Industries, Millville, N.J.) in Dubos broth base without polysorbate 80 (GIBCO Diagnostics, Madison, Wis.) (adjusted to pH 7.2 before autoclaving) and 20% (vol/vol) Dubos Medium Albumin (Difco Laboratories, Detroit, Mich.). Complete medium is referred to as Dubos-albumin (DA). The majority of tissue debris was removed by low-speed centrifugation ($108 \times g$, 5 min, 10°C) in disposable 15-ml polypropylene tubes. The bulk of soluble tissue components was removed by centrifugation ($2,710 \times g$, 45 min, 10°C) of the supernatant and resuspension of the bacterial pellet in 5 ml of DA. Samples (100 μl) were removed for cell counts and for contamination check on blood agar plates, Middlebrook 7H11 and Lowenstein-Jensen slants, Trypticase soy broth, and thioglycolate broth (all from BBL Microbiology Systems, Cockeysville, Md.). Media were incubated at 33°C, and the *M. leprae* suspensions were held at 4°C. Media were observed after 24 h and then incubated for up to 1 month. If all media were negative for growth at 24 h, the *M. leprae* suspension was used as is. If low-level contamination was evident or suspected, ampicillin, amphotericin B, or both were added to the suspension to achieve final concentrations of 50 (ampicillin) and 2.5 (amphotericin B) μg/ml in the culture medium. These antibiotics have shown no effect on ATP levels of *M. leprae* in repeated tests or on viability in the mouse footpad (18).

Incubation conditions and ATP assay. The *M. leprae* suspension was diluted in fresh DA to a density of 1.5×10^7 to 8×10^7 cells per ml. Samples (1 ml) were distributed to sterile 1.5- or 2.0-ml screw-cap microfuge tubes or, in some cases, vented tubes (16 by 100 mm) or 2-ml tissue culture wells. All antimicrobial agents were obtained from Sigma Chemical Co., St. Louis, Mo., except clofazimine (CIBA-GEIGY Corp., Summit, N.J.), ethionamide (Ives Laboratories, Inc., New York, N.Y.), and ciprofloxacin (Miles Laboratories, Inc., Elkhart, Ind.). Compounds were prepared at 100× concentrations, filter sterilized, and added in a volume of 10 μl. Tubes were incubated at 33°C.

At appropriate intervals, replicate samples were centrifuged ($12,096 \times g$, 5 min), washed twice in 10 mM Tris–1 mM EDTA (pH 7.7), and suspended in 0.1 ml of the same solution. A 10-μl quantity was removed, diluted 1:10 in phosphate-buffered saline buffer, and counted by the technique of Shepard and McRae (20). Chloroform (40 μl) was added to the remaining suspension, and the tubes were sealed and vortexed briefly. Samples were heated at 100°C for 9 min in a heat block to release intracellular ATP. After cooling on ice, they were heated at 100°C under vacuum for 1 min and cooled on ice, and the ATP was solubilized in 1.6 ml of deionized water.

ATP assays were done essentially as described by Kvach et al. (13), except that 0.1 ml of luciferin-luciferase was

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TABLE 1. In vitro effect of antileprosy drugs on intracellular ATP of *M. leprae*

Drug	Concn (μg/ml)	Mean (SD) pg of ATP/10^6 cells[a] at day:		
		7	14	21
Control	0	186 (42)	69 (25)	56 (2)
Rifampin	0.2	141 (7)	34 (2)	7 (3)**
	2.0	166 (73)	23 (14)***	13[b]
	20.0	240 (55)	16 (10)***	5 (2)*
Dapsone	0.2	179 (38)	55 (17)	63 (32)
	2.0	135 (22)	40 (4)	42 (37)
	20.0	116 (15)	62 (9)	48 (26)
Ethionamide	0.2	129 (22)	51[b]	ND[c]
	2.0	45 (5)	39 (10)	32 (8)***
	20.0	36 (5)***	14 (8)***	11 (3)*
Clofazimine	0.2	172 (70)	56 (7)	48 (16)
	2.0	119 (17)***	10 (4)***	7[b]
	20.0	86 (9)**	6 (1)***	5 (8)**

[a] Data are given as the mean (standard deviation) of triplicate or duplicate samples. *, $P < 0.001$; **, $P < 0.005$; ***, $P < 0.05$.
[b] Single sample value.
[c] ND, Not done.

injected into 0.4-ml portions of the test samples. An Aminco Integrator-Timer (American Instrument Co., Silver Spring, Md.) was used for determining the relative intensity values. Appropriate ATP standards, stored at −80°C, were used to construct a standard curve immediately after the measurement of bacterial ATP. Cell counts were normally determined only for zero-time samples such that all ATP values correspond to picograms per 10^6 cells based on the zero-time counts. Data were analyzed by the two-tailed Student *t* test.

RESULTS

Effect of antileprosy compounds. In at least 10 distinct experiments, control cells (in drug-free media) consistently demonstrated a decay in intracellular ATP over a 3- to 4-week period. Although this rate varied among individual experiments, it was sufficiently slow to allow for detection of accelerated decay rates in the presence of antileprosy agents. Rifampin-treated cells usually showed an initial dose-related (but nonsignificant) increase in ATP pools at 7 days but thereafter exhibited an accelerated rate of ATP decay compared with controls at doses ranging from 0.2 to 20 μg/ml (Table 1). The latter effect was observed in five of five individual experiments. Occasionally, significant activity was not observed until 21 days postincubation. In contrast to rifampin, dapsone did not elicit such a change in ATP pools at any concentration. Ethionamide-treated cells showed an accelerated ATP decay as early as 7 days postincubation in repeated experiments but only at ≥20 μg/ml. *M. leprae* responded in a similar manner to clofazimine (B663), showing an early accelerated ATP decay, but in this case, cells were sensitive to ≥2 μg/ml.

Comparative activity of clofazimine analogs. Two clofazimine analogs, B3785 and B826, were synthesized by and obtained from J. F. O'Sullivan, Trinity College, Dublin, Ireland. These analogs were found to be the most active among a number of compounds when evaluated against clofazimine-susceptible and -resistant *Mycobacterium smegmatis* in vitro and also showed roughly equivalent activity to B663 against *M. leprae* in a single mouse footpad experiment (J. F. O'Sullivan, personal communication). The activity of these three compounds was compared at four concentrations, ranging from 0.031 to 2.0 μg/ml, in a kinetic study in the ATP system.

After 6 days of incubation, all three compounds at 2 μg/ml elicited a significant reduction in ATP pools compared with control cells (Fig. 1). However, B3785 and B826 also showed strong activity at 0.5 μg/ml, whereas B663 had no detectable effect. At 10 days (data not shown) postincubation, B663 still showed activity only at 2 μg/ml, whereas B3785 and B826 caused significant rates of ATP decay at 0.125 μg/ml.

Although all three compounds caused a significant reduction in ATP pools at 0.125 μg/ml by day 20, only B3785- and B826-treated cells showed activity at 0.031 μg/ml ($P < 0.01$).

These results were confirmed in a second experiment (data not shown) in which assays were conducted at a single time point, 21 days postincubation. All compounds were active at 2 μg/ml and inactive at 0.02 μg/ml. At 0.2 μg/ml, B663 was inactive, whereas B3785 and B826-treated cells had ATP levels significantly lower than both controls and B663-treated cells ($P < 0.01$).

In vitro effect of established antimicrobial agents: preliminary results. A number of antimicrobial agents were screened for activity in the in vitro system by measuring ATP levels of control and drug-treated cells at a single time point, 20 to 21 days postincubation. Compounds were added at final concentrations of 0.2, 2.0, and 20 μg/ml, except for cell wall synthesis inhibitors, which were added at 2.0, 20, and 200 μg/ml.

The effects of a number of antibiotics known to inhibit protein synthesis on 70s ribosomes are shown in Table 2. Minocycline appeared to be more active than tetracycline, and chloramphenicol and erythromycin showed very strong activity, the latter at both 2 and 20 μg/ml. Subsequent

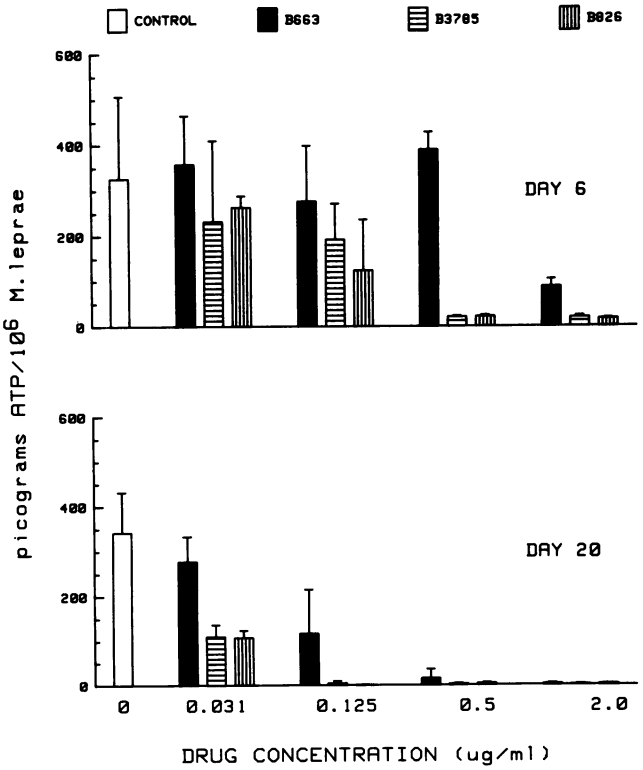

FIG. 1. Comparative in vitro effect of clofazimine analogs on intracellular ATP of *M. leprae*. Cells were incubated in DA (pH 7.2) or in medium containing various concentrations of B663, B3785, or B826. Cells were analyzed for intracellular ATP at 6 and 20 days postincubation. Error bars represent one standard deviation.

experiments with erythromycin have confirmed its strong activity.

Among the cell wall synthesis inhibitors, neither the beta-lactams nor the peptide antibiotics were active at 2 or 20 μg/ml. The comparative activity of these agents at 200 μg/ml is shown in Table 3. Although the beta-lactamase-sensitive and -resistant penicillins did not show activity, cephalothin and cycloserine exhibited significant activity. A second experiment with cloxacillin and cycloserine confirmed these results, although cycloserine appeared somewhat less active.

Finally, a number of antimicrobial agents with various modes of action were tested (Table 4). None of these agents was active at 0.2 μg/ml. At 20 μg/ml, neither isoniazid nor trimethoprim demonstrated activity. Of the nucleic acid synthesis inhibitors, neither the antifungal agent, griseofulvin, nor the quinolone, nalidixic acid, was active, whereas ciprofloxacin, one of the new class of fluoroquinolones, showed weak but significant activity. Of the inhibitors of cytoplasmic membrane function, the gramicidins appeared to have weak but significant activity.

DISCUSSION

Three of the four most commonly used antileprosy compounds demonstrated activity in this system. The rapid activity of clofazimine, also described by Kvach et al. (13) in phosphate buffer, could be caused by a direct action on the respiratory chain. The initial increase in ATP pools in response to rifampin may be caused by a decline in ATP-consuming anabolic functions. The failure of dapsone to show activity may represent the major disadvantage of this system: an insensitivity to compounds affecting specific enzymes involved in pathways which would not be expected to affect ATP levels within the available time frame.

The sensitivity of the system to agents with various modes of action is encouraging. The activity of chloramphenicol at 20 μg/ml is consistent with a report on the in vitro inhibition of protein synthesis in armadillo-derived *M. leprae* by this drug (12). The strong activity of minocycline is supported by a recent report on the impressive activity of this drug against *M. leprae* in vivo (10). Although erythromycin is known to have antimycobacterial activity (16), we are not aware of any reports involving *M. leprae*. There may be a rough correlation between the in vitro activity of cycloserine only at the very high concentration of 200 μg/ml and its in vivo activity in the mouse footpad only at dietary concentrations of >0.5% (wt/wt) (9). Ciprofloxacin has demonstrated activity against a number of clinically important mycobacteria (8). Its mild in vitro activity against *M. leprae* suggests that other fluoroquinolones should be evaluated. To our knowledge, there are no reports on activity of gramicidins on *M. leprae*. The lack of activity with isoniazid and trimethoprim is consistent with previous reports in the mouse footpad assay (11, 18, 19).

The key to successful implementation of the system described above is the use of an inoculum with a sufficiently high level of viability such that accelerated rates of ATP decay can be detected. Inocula prepared from athymic, nude mouse footpads, containing $\leq 10^{10}$ *M. leprae* cells, had initial ATP levels of 200 to 1,000 pg/10^6 cells. The contribution of host ATP from residual debris was judged to be negligible based on relative values obtained from uninfected footpads processed in an identical manner. These values are similar to those found by Kvach et al. for *M. leprae* purified from armadillo liver (13). Although these inocula were sufficiently viable for the in vitro system, bacilli harvested from more advanced infections usually had a much lower overall viability and a corresponding faster ATP decay when incubated in vitro. Such footpads can most likely be used by enriching for viable cells on continuous (13) or two-step discontinuous

TABLE 2. In vitro effect of protein synthesis inhibitors on intracellular ATP of *M. leprae*

Drug (no. of samples)	Concn (μg/ml)	Mean (SD) pg of ATP/10^6 cells[a]
None (8)	0	311 (53)
Erythromycin (2)	0.2	132 (26)**
	2.0	21 (6)*
	20.0	17 (0.5)*
Chloramphenicol (3)	0.2	332 (38)
	2.0	287 (57)
	20.0	30 (1)*
Minocycline (3)	0.2	324 (19)
	2.0	123 (82)*
	20.0	29 (3)*
Tetracycline (3)	0.2	285 (52)
	2.0	298 (3)
	20.0	198 (65)***

[a] At 3 weeks postincubation in DA. *, $P < 0.01$; **, $P = 0.002$; ***, $P = 0.018$.

TABLE 3. In vitro effect of cell wall synthesis inhibitors on intracellular ATP of *M. leprae*

Drug[a] (no. of samples)	Mean (SD) pg of ATP/10^6 cells[b]
None (17)	127 (44)
Cycloserine (3)	21 (15)*
Cephalothin (3)	27 (6)*
Bacitracin (3)	92 (2)
Penicillin G (3)	98 (35)
Ampicillin (3)	100 (72)
Methicillin (3)	126 (14)
Cloxacillin (3)	140 (23)

[a] Final concentration of 200 μg/ml. No activity was observed at 2 or 20 μg/ml.

[b] At 3 weeks postincubation in DA. *, $P < 0.001$.

TABLE 4. In vitro effect of antimicrobial agents with various modes of action on intracellular ATP of *M. leprae*

Drug (no. of samples)	Concn (μg/ml)	Mean (SD) pg of ATP/10^6 cells[a]
None (18)	0	110 (27)
Gramicidin (3)	2	94 (40)
	20	28 (13)*
Gramicidin S (3)	2	92 (76)
	20	70 (17)***
Polymyxin B (3)	2	103 (36)
	20	116 (27)
Ciprofloxacin (3)	2	67 (26)***
	20	60 (13)**
Nalidixic acid (3)	2	92 (37)
	20	94 (7)
Griseofulvin (3)	2	98 (15)
	20	101 (26)
Trimethoprim (3)	2	90 (37)
	20	88 (27)
Isoniazid (3)	2	89 (78)
	20	129 (44)

[a] At 3 weeks postincubation in DA. *, $P < 0.001$; **, $P = 0.006$; ***, $P < 0.03$.

(unpublished observations) Percoll gradients; however, use of viable nonpurified suspensions was preferred here for the sake of simplicity.

The use of 1-ml samples in microfuge tubes allowed for the processing of the entire sample and thereby eliminated problems associated with clumping when subsamples are drawn. The use of a single tube for incubation and processing allows for the analysis of up to 45 samples in a day by two people. The total cost of materials (all disposable) is less than $1.00 per test sample, exclusive of the cost of propagating the *M. leprae* inoculum in the nude mouse.

This system appears to be most useful as a primary screen for testing large numbers of new compounds or for comparing analogs of compounds known to have activity in the in vitro ATP system. Active compounds ultimately need to be confirmed by the mouse footpad technique.

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ADDENDUM IN PROOF

Recent studies in our laboratory have shown that the maintenance of ATP levels in *M. leprae* is markedly enhanced in Percoll-purified cells incubated under altered biophysical conditions. Under these conditions, dapsone (2 μg/ml) effects a significantly accelerated rate of ATP decay after 2 to 3 weeks of incubation compared with that in drug-free cells.

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