Inhibition of Phenolic Glycolipid-I Synthesis in Extracellular *Mycobacterium leprae* as an Indicator of Antimicrobial Activity[1,2]

Eugene B. Harris, Scott G. Franzblau, and Robert C. Hastings[3]

Previous efforts to study the effect of drugs on the metabolism of *Mycobacterium leprae* have been hindered by the inability to culture the organisms on artificial media. The mouse foot pad (MFP) technique is the most common method for evaluating drug susceptibility in *M. leprae* ([19]). This technique involves measuring the ability of a drug administered to a mouse to inhibit the limited multiplication of *M. leprae* inoculated into the mouse's foot pad. The principal disadvantages of this technique are its cost, the time required (6–9 months) for evaluating a drug's effectiveness, and the necessity for the drug to have favorable pharmacokinetics in the mouse in order to show activity. This has prompted the recent development of a number of *in vitro* systems ([11]) that rely on inhibition of bacterial metabolic functions as indices of drug activity. We have previously demonstrated the potential of adenosine-5-triphosphate (ATP) measurement ([3]) and the radio-respirometric determination of palmitate oxidation ([1]) to rapidly assess drug susceptibility in *M. leprae*. However, due to the possible presence of other bacteria in infected armadillo tissues and human leprosy tissues, it would be of value to develop, in addition, an assay based on an *M. leprae*-specific metabolite.

Within the past few years, an *M. leprae*-specific glycolipid, phenolic glycolipid-I (PGL-I), has been identified and characterized ([13]). Ramasesh, *et al.* ([16]) have recently reported the incorporation of [U^{14}C] palmitic acid ([U^{14}C] PA) into the PGL-I fraction of *M. leprae* residing in cultured mouse peritoneal macrophages. This procedure has subsequently been adapted for drug sensitivity studies (Ramasesh, *et al.*, unpublished data).

We have observed a linear incorporation of [U^{14}C] PA into the PGL-I of extracellular *M. leprae* as well ([5]). This simplified incubation system was sensitive to biophysical perturbations ([2]) and to the antileprosy agents dapsone and rifampin. With inhibition of PGL-I synthesis as the criterion of drug susceptibility, an expanded series of established antimicrobials were tested for activity against *M. leprae*.

MATERIALS AND METHODS

***M. leprae* suspension.** Freshly harvested organisms from experimentally infected foot pads of nude mice (HSD athymic nu/nu/AF; Harlan Sprague-Dawley, Inc., Indianapolis, Indiana, U.S.A.) were prepared as previously described ([1]) in Dubos broth base without polysorbate 80 (Gibco Diagnostics, Madison, Wisconsin, U.S.A.) without pH adjustment and containing 20% (v/v) Dubos medium albumin (Difco Laboratories, Detroit, Michigan, U.S.A.). The medium is referred to as DA. Briefly, the procedure involves initial surface decontamination of the foot pads by ultraviolet irradiation, an ethyl ether wash, Acudyne (Acme United Corp., Bridgeport, Connecticut, U.S.A.) treatment, and a 70% ethanol rinse. The foot pads were then minced and homogenized in DA. A slow-speed centrifugation (100 × *g* for 5 min) removed most of the tissue debris. The organisms were then pelleted from the supernatant by centrifugation at 2700 × *g* for 45 min. The final bacterial pellet was suspended in the desired volume of medium and enumerated. Ampicillin (50 μg/ml) and amphotericin B (2.5 μg/ml) were

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[3] E. B. Harris, B.S., Research Chemist, Biochemistry Research Department; S. G. Franzblau, Ph.D., Microbiologist, Pharmacology Research Department; R. C. Hastings, M.D., Ph.D., Chief, Laboratory Research Branch, GWL Hansen's Disease Center, Carville, Louisiana 70721, U.S.A.

added to the medium as protection against any low-level contamination that might be present. Any contaminant in the suspension might rapidly overgrow *M. leprae* and compete for the ^{14}C-labeled substrate. Previous experimental work has shown that these compounds have no effect on *M. leprae* [3, 18].

Antimicrobials. All antimicrobials were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A., except: ciprofloxacin (Miles Laboratories, West Haven, Connecticut, U.S.A.); clofazimine (CIBA-GEIGY, Summit, New Jersey, U.S.A.) and rifabutin (Farmitalia Erba, Milan, Italy).

***In vitro* susceptibility testing.** Suspensions of *M. leprae* were diluted in DA containing ampicillin (50 μg/ml) and amphotericin B (2.5 μg/ml) to a concentration of 1×10^8 bacilli per ml, and 6 ml aliquots were distributed into 12-ml screw-cap tubes. Drugs were added at the appropriate concentrations, and the cultures were subdivided into three 2-ml aliquots. Medium containing bacilli but devoid of antimicrobial agents served as controls. The tubes were tightly capped and incubated at 33°C for 4 days, after which 0.5 μCi of [$U^{14}C$] PA (850 mCi/mmol; New England Nuclear, Boston, Massachusetts, U.S.A.) was added to each tube. The reaction tubes were then tightly capped and incubated at 33°C for an additional 8 days.

PGL-I extraction and quantitation. PGL-I was recovered from the lyophilized samples by a procedure adapted from Hunter and Brennan [13] and slightly modified by Franzblau, *et al.* [5]. In brief, the total lipid extracts from the lyophilized samples were subjected to a biphasic wash, and the glycolipid fractions in the lower organic layers were collected by methanolic chloroform elution from silicic acid : celite columns. After further separation by thin-layer chromatography (TLC), the areas corresponding to the PGL-I were identified, removed by scraping from the TLC plates, and their radioactivity measured.

RESULTS

The results of the *in vitro* drug-sensitivity testing are presented in The Table. Control values in four individual experiments ranged from 2.2–8.2 pico moles of [$U^{14}C$] PA incorporated per 2×10^8 *M. leprae.* As determined by [$U^{14}C$] PA incorporation into the PGL-I fraction, there was decreased synthesis of the glycolipid in the presence of the most frequently used antileprosy drugs dapsone, rifampin, and clofazimine. On the other hand, the antileprosy drug ethionamide at 2 μg/ml caused an increase in PGL-I synthesis.

Among the protein synthesis inhibitors, erythromycin and chloramphenicol were highly active and minocycline, streptomycin, and clindamycin also showed significant inhibition. Cephalothin alone was ac-

THE TABLE. *Effect of antimicrobial agents on incorporation of [$U^{14}C$] palmitate into PGL-I fraction of* M. leprae.

Antimicrobials	% Inhibition[a] at drug concentration of	
	2 μg/ml	20 μg/ml
Clinical antileprosy		
Dapsone	87[b] (6)[c]	ND[d]
Rifampin	65[b] (10)	ND
Clofazimine	48[e] (16)	ND
Ethionamide	+[f]	ND
Protein synthesis inhibitors		
Erythromycin	77[g] (4)	88[b] (5)
Chloramphenicol	66[e] (22)	80[g] (9)
Minocycline	+	72[b] (6)
Tetracycline	+	+
Streptomycin	26[e] (7)	42[e] (8)
Gentamicin	7 (6)	33 (15)
Clindamycin	32 (12)	60[g] (7)
Cell wall synthesis inhibitors		
Cephalothin	43[e] (11)	56[b] (3)
Cloxacillin	13 (14)	24 (9)
Cycloserine	3 (1)	4 (3)
Bacitracin	40 (15)	10 (9)
Nucleic acid synthesis inhibitors		
Ciprofloxacin	46[g] (8)	71[b] (1)
Nalidixic acid	14 (8)	66[g] (2)
Rifabutin (LM 427)	+	43 (5)
Griseofulvin	52[e] (11)	45 (17)
Miscellaneous		
Cerulenin	76[g] (7)	92[b] (8)
Isoniazid	35 (19)	77[g] (5)
Polymyxin B	0	0

[a] Controls and antibiotic activities were determined in triplicate and the results averaged.
[b] $p < 0.01$ compared to control values, *t* test.
[c] Numbers in parentheses represent standard deviations.
[d] ND = not done.
[e] $p < 0.05$ as compared to control values, *t* test.
[f] 1.2–1.9-fold increase in PGL-I incorporation as compared to control values.
[g] $p < 0.02$ compared to control values, *t* test.

tive among the inhibitors of cell wall synthesis. The quinolones ciprofloxacin and nalidixic acid demonstrated activity, but the results with griseofulvin were inconclusive. Cerulenin was highly active as was isoniazid at the higher concentration.

DISCUSSION

In general, those compounds inhibiting PGL-I synthesis have also been active in the *in vitro* ATP system ([3]) and/or in the traditional MFP system ([6–8, 11, 12, 17–19]). In all three systems minocycline is active while tetracycline and cycloserine are inactive or weakly active. Streptomycin and clindamycin are bacteriostatic in the MCP and weakly active in the PGL-I system, while gentamicin had questionable activity in both systems.

Erythromycin and ciprofloxacin are active *in vitro* but inactive in the MFP. It is likely that the poor pharmacokinetic properties of these compounds in mice are responsible for their lack of *in vivo* efficacy. Erythromycin could not be detected in the serum of mice receiving erythromycin ethylsuccinate at 0.1% w/w in the diet ([4]). Pefloxacin, which reaches and maintains higher serum concentrations than ciprofloxacin in mice, is active in suppressing the growth of *M. leprae* in the MFP ([10]).

In several instances the presence of the drug at a concentration of 2 μg/ml (minocycline, tetracycline, and ethionamide) resulted in the stimulation of PGL-I synthesis. In the case of minocycline, a 10-fold increase in drug concentration resulted in inhibition. Low concentrations of various drugs have been reported to cause morphological changes in cell walls, including increased synthesis of exopolysaccharides ([9, 14]).

The antibiotic cerulenin acts upon the condensation of acyl-acyl carrier protein and malonyl-acyl carrier protein (ACP), a vital step in the biosynthesis of fatty acids ([15]). The almost complete inhibition of PGL-I synthesis in the presence of this compound indicates that *M. leprae* is actively synthesizing PGL-I *in vitro*. Cerulenin probably acts in this system by inhibiting a specific ACP-dependent elongation system.

It is recognized that the use of albumin in the medium may result in some degree of drug-protein binding, thereby reducing drug availability and activity. In this respect, the effective *in vitro* concentrations of the various drugs tested may not be a true reflection of free drug concentrations under *in vivo* conditions. Additionally, there is a possibility that amphotericin B and ampicillin, present in the medium throughout the testing procedure, may exert some degree of antimicrobial synergism. However, their use does not negate the usefulness of this procedure, rather it emphasizes the need to approach the screening of antileprosy drugs with caution.

From the data provided by this study, it appears that the synthesis of the *M. leprae*-specific phenolic glycolipid can be exploited as a relatively rapid *in vitro* assay for drug susceptibility.

SUMMARY

The effects of 22 antimicrobial agents on the incorporation of [U^{14}C] palmitic acid ([U^{14}C] PA) into the unique phenolic glycolipid-I (PGL-I) antigen of *Mycobacterium leprae* were studied. Nude-mouse-propagated *M. leprae* were incubated in a modified Dubos medium in the presence of antimicrobial agents for 4 days. [U^{14}C] PA was then added and incubation was continued for 8 days. The antileprosy agents dapsone, rifampin, and clofazimine (2 μg/ml each) caused a significant reduction in [U^{14}C] PA incorporation into PGL-I. Among other agents, the most active were erythromycin, chloramphenicol, and cerulenin. Low concentrations of ethionamide, tetracycline, and minocycline stimulated label incorporation. This system may prove useful in the evaluation of antileprosy agents.

RESUMEN

Se estudiaron los efectos de 22 agentes antimicrobianos sobre la incorporación del ácido palmítico marcado con ^{14}C [(U^{14}C)PA] en el glicolípido fenólico-I (GLF-I) del *Mycobacterium leprae*. El *M. leprae* propagado en ratones desnudos se incubó en un medio de Dubos modificado en presencia de los agentes antimicrobianos durante 4 días. Enseguida se adicionó (U^{14}C)PA y la incubación se continuó por 8 días. Los agentes antileprosos dapsona, rifampina, y clofazimina (2 μg/ml de cada uno) causaron una reducción significante en la incorporación de (U^{14}C)PA en el GLF-I. Entre otros agentes, los más activos fueron la eritromicina, el cloranfenicol, y la cerulenina. La etiona-

mida, la tetraciclina, y la minociclina estimularon la incorporación de la marca. Este sistema podría resultar útil en la evaluación de agentes antileprosos.

RÉSUMÉ

On a étudié les effets de 22 agents antimicrobiens sur l'incorporation de l'acide palmitique marqué par l'U^{14}C (U^{14}C PA) dans l'antigène spécifique PGL-I de *Mycobactérium leprae*. Des bacilles de la lèpre propagés chez la souris glabre ont été incubés pendant 4 jours dans un milieu de Dubos modifié, en présence de ces agents antimicrobiens. L'U^{14}C PA a alors été ajouté, et on a poursuivi l'incubation pendant 8 jours. La dapsone, la rifampicine, et la clofazimine (à la dose de 2 μg/ml pour chacun de ces médicaments antilépreux), a entraîné une réduction significative dans l'incorporation de l'U^{14}C PA dans le PGL-I. Parmi les autres composés étudiés, les plus actifs se sont révélés être l'érythromycine, le chloramphénicol, et la cérulénine. Des concentrations faibles d'éthionamide, de tétracycline, de minocycline, ont stimulé l'incorporation du produit marqué. Ce système pourrait être utile pour évaluer les médicaments antilépreux.

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