Activities of Fluoroquinolone, Macrolide, and Aminoglycoside Drugs Combined with Inhibitors of Glycosylation and Fatty Acid and Peptide Biosynthesis against *Mycobacterium avium*

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Smooth- and rough-colony variants of *Mycobacterium avium* serovar 4 were treated with three classes of drugs. The drugs were chosen for their potential inhibitory effects on the biosynthesis of the cell envelope-associated serovar-specific glycopeptidolipid antigens. Growth was monitored radiometrically with a BACTEC 460-TB instrument, and MICs were determined for each drug. Both variants were then treated with inhibitory drugs in combination with antimicrobial agents that have demonstrated effectiveness against *M. avium*. No growth inhibition was observed with 6-fluoro-6-deoxy-D-glucose or avidin. Inhibitors of glycosylation, i.e., 2-deoxy-D-glucose, bacitracin, and ethambutol, were inhibitory to smooth- and rough-colony variants, whereas drugs that inhibit peptide synthesis, i.e., N-carbamyl-L-isoleucine and m-fluoro-phenylalanine, were more inhibitory for the rough-colony variant. Cerulenin, which affects fatty acid synthesis, was inhibitory for both variants, but it appeared to be more effective at inhibiting the growth of the smooth-colony variant at equivalent concentrations. Generally, when inhibitors of glycosylation were used with sparfloxacin and amikacin, a synergistic effect was observed for only the smooth variant. When drugs that affect peptide synthesis were used in combination with amikacin, a synergistic effect was observed for the rough variant, and when cerulenin was used in combination with sparfloxacin or amikacin, a synergistic effect was observed for both variants. Lipid analysis revealed that although the rough variant lacks the serovar-specific glycopeptidolipid antigens, it does possess a group of phenylalanine-isoleucine-containing lipopeptides that may explain its different susceptibility patterns to m-fluoro-phenylalanine and N-carbamyl-L-isoleucine. The significance of these results is discussed with reference to various components in the cell envelope and their importance in cell wall permeability.

The new emphasis of the National Institute of Allergy and Infectious Diseases is "to spark efforts" to better understand the opportunistic infections associated with AIDS so that new and effective treatments can be developed (45). A major obstacle preventing the development of better therapies for those opportunistic pathogens is a "basic lack of knowledge about the pathogens that cause such diseases" (45). The *Mycobacterium avium* complex represents one of the most important groups of opportunistic pathogens infecting patients with AIDS (23, 45). Because of the clinical relevance of the *M. avium* complex in the management of AIDS, a renewed interest in antimycobacterial therapy has taken place. Despite the *M. avium* drug susceptibility observed in vitro, successful therapy, even with multiple-drug regimens, appears to be limited (16, 46, 51) except in some reported trials (1, 20). Even so, other trials have had to be discontinued because of drug toxicity (8), a problem that must be considered when treating patients with AIDS (19). One explanation for the in vivo drug resistance in clinical and animal trials is the refractory nature of the *M. avium* complex that may result from the cell wall matrix (10, 33, 36). Although a specific cell wall component has not been attributed to decreased drug permeation, there is suggestive evidence that several components may play a role in that phenomenon.

Rastogi et al. (33) proposed that a polysaccharide outer layer may be responsible for the impermeability of smooth-transparent variants to various substrates and drugs and also increased pathogenicity. The presence of the fibrillar material described by Draper (13) and Kim et al. (21) and later referred to as the superficial L_1 layer (2) may also be important as a barrier because of the polar glycopeptidolipids (GPLs) that make up its superficial location (4, 6, 44). Crowle et al. (9) suggested that both the polysaccharide layer (32, 33) and the GPL layer (6, 44) may be instrumental as drug barriers for *M. avium* growing within macrophages.

The inertness of GPLs to macrophage degradation has been reported for *M. avium* serovars 4 (47) and 20 (18). This would suggest that if the GPLs were contributing toward a permeability barrier, their accumulation during postphagocytic events could result in an increased drug resistance for intramacrophagic *M. avium*. Deposits of GPLs (or C-mycosides) have been reported in cell cultures (6, 13, 21) or animals (14, 17) by various laboratories, the most recent of which was by Roulong et al. (41), who demonstrated its deposition in phagosomes of murine macrophages by means of freeze fracture analysis. Of interest in the study of Roulong et al. (41) is the fact that the accumulation of GPLs was not observed on either extracellular *M. avium* or intramacrophagic *M. avium* after short-term (45-min) inoculation of mice, but was observed only after lengthy infection of up to 3 months. These studies support our previous immunocy-

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tochemical observations (44) and suggest that GPLs accumulate within the phagolysosomal compartment in juxtaposition to the mycobacterial cell and the phagolysosomal membrane. Their accumulation may not only protect the organism from macrophage degradation but they may also impede the penetration of drugs, thus allowing the organism to be more refractory to antimycobacterial agents (9, 41).

To better understand the contribution of GPLs to drug resistance, potential inhibitors of GPL biosynthesis were selected in an attempt to define potential drug targets. Because the GPLs comprise three major constituents, carbohydrate, lipid, and peptide, we examined the effects of three classes of inhibitors on the growth of *M. avium* serovar 4. Both a smooth-colony variant, which synthesizes the GPL (25, 47, 49), and its rough-colony variant, which lacks an ability to synthesize the GPL, were treated with drugs that inhibit glycosylation and fatty acid and peptide biosynthesis. Once the MICs were determined for each drug, the use of those inhibitory drugs and antimicrobial agents in combination was examined in an effort to help identify potential metabolic targets for treatment of *M. avium* infections. In addition, lipids from the rough variant were examined for the presence of lipopeptides that might suggest the increased susceptibility of the rough variant to drugs that inhibit peptide synthesis.

MATERIALS AND METHODS

Mycobacteria. *M. avium* complex serovar 4 (TMC 1463), smooth-colony parent strain, was provided by the National Jewish Hospital and Research Center (Denver, Colo.) through Darrel Gwinn, National Institute of Allergy and Infectious Diseases (Bethesda, Md.). The rough variant of serovar 4 was developed by means of the slant-tube culture procedure reported previously (5). Mycobacteria were cultivated in 7H9 Middlebrook broth or agar (Difco Laboratories, Detroit, Mich.) which was supplemented with glycerol and oleic acid-albumin-dextrose (OADC) (Difco) as described previously (47). In the case of mycobacteria cultured in broth, growth was monitored on a Klett-Summerson spectrophotometer with a no. 42 filter (47).

Internal radiolabeling of mycobacterial lipids. Mycobacteria were cultured in 7H9 Middlebrook broth containing glycerol and OADC as described previously (49). When cultures reached the exponential phase (100 to 150 Klett units), either L-[U-^{14}C]phenylalanine ([^{14}C]Phe; 405 mCi/mM) or L-[U-^{14}C]isoleucine ([^{14}C]Ile; 166 mCi/mM) (ICN Radiochemicals, Inc., Irvine, Calif.) was added at a concentration of 0.2 μCi/ml. To ensure optimum dispersal of rough variants, glass beads (3-mm diameter) were added to the culture flasks. Mycobacteria were autoclaved and harvested once they had reached 450 to 500 Klett units (10 to 12 days) and were then lyophilized and stored at −20°C until the lipids were extracted (18, 47, 49).

Extraction and purification of lipids. Lipids were extracted from lyophilized mycobacteria with chloroform-methanol (2:1) and were processed by the Folch procedure as described previously (18, 47, 49). Following extraction, lipid fractions were applied, in parallel, to thin-layer chromatographic (TLC) plates at concentrations ranging from 100 to 300 μg and were then developed in either chloroform-methanol-water (60:12:1) (solvent A) or chloroform-methanol (11:1) (solvent B). Following development in the appropriate solvent, individual lipid components were first localized by exposing the TLC plate to iodine vapors. Sections of 1 cm were then scraped into vials, and the

radioactivity was measured in EcoLite scintillation fluid (WestChem). Subsequently, the TLC plate was sprayed with an orcinol-sulfuric acid reagent to confirm the presence or absence of GPLs in the remaining unscraped, adjacent lanes.

Chemical procedures. For amino acid analysis, lipid components were hydrolyzed in 6 N HCl, and the resulting acid hydrolysates were examined for amino acids by development on cellulose TLC plates in butanol-acetic acid-water (40:10:10; solvent C) and detection with a 0.2% ninhydrin acetone solution (18). The distribution of radioactivity was determined by counting 1-cm sections of the TLC plate in EcoLite scintillation fluid, and appropriate amino acid standards were run in parallel to identify the amino acids. Quantitation of amino acids was achieved with a Dionex D-300 amino acid analyzer. Carbohydrate was estimated by means of the orcinol-sulfuric acid procedure (12). Some lipid fractions were treated with 0.2 N methanolic NaOH for 30 min at 37°C (6, 7) to remove contaminating fatty acids (7).

HPLC separation of lipid samples. Lipids were purified by high-performance liquid chromatography (HPLC) by using a Beckman System Gold model 126 programmable solvent delivery module controlled by System Gold chromatography software. Samples were applied to an Ultrasphere 5-μm Spherical 8.0-nm-pore-size SI analytical column (4.6 by 250 mm) attached to a guard column containing the same solid support (4.6 by 45 mm) by using Beckman System Gold chromatography software for analysis, a model 166 programmable variable UV detector (257 nm), and a 171 solid-system radioisotope detector for detection of lipid components. Lipid samples were injected at concentrations ranging from 200 to 800 μg and were separated in a mobile phase of 100% chloroform for 10 min and then a 40-min gradient of 0 to 10% methanol in chloroform at a flow rate of 1.0 ml/min (43, 49).

Growth conditions for inhibitory studies. Mycobacterial growth was monitored radiometrically by means of a BACTEC 460-TB instrument (Becton Dickinson, Towson, Md.) as reported previously (35, 36). Growth, which was in a confined atmosphere containing ^{14}C-labeled palmitate in Middlebrook 7H12a broth, was measured as a function of the release of ^{14}C-labeled CO_2. The released CO_2 was then captured by a detector, and growth was expressed as a numerical value, referred to as the growth index (GI), which ranged from 1 to 999.

For experimental procedures, a vial containing 4 ml of broth (primary culture) was inoculated with 0.1 ml of mycobacterial suspension (optical density of 0.15 at 650 nm, measured with a Coleman Junior II spectrophotometer). Once the primary culture had reached a GI of 500, a portion was removed and diluted 10-fold and 0.1 ml of this diluted portion was injected directly into drug-containing vials. In the case of cerulenin testing with the rough variant, vials were inoculated directly from the primary culture (explained below). MICs were interpreted as reported earlier (35, 36) by comparing the GIs of the drug-containing vials with those of their respective controls once the GI in the control vial reached a value of 30 or more. The method for MIC determinations, using the 1% proportion criterion for bacterial growth, has previously been described in detail (35).

Combined drug (plus inhibitor) action was investigated radiometrically by the previously described x/y quotient calculation method (36). For this purpose, all the drugs and inhibitors were used at sublethal concentrations. The drugs used were ciprofloxacin (0.05 μg/ml), sparfloxacin (0.05 μg/ml), clarithromycin (0.05 μg/ml), and amikacin (0.5 μg/ml), whereas the inhibitors used included bacitracin (5 μg/ml), N-carbamyl-L-isoleucine (100 μg/ml), 2-deoxy-D-

glucose (500 μg/ml), cerulenin (0.5 μg/ml), m-fluoro-phenyl-alanine (100 μg/ml), and ethambutol (1 μg/ml).

For x/y quotient determination, a bacterial preculture with a GI of 500 was diluted 10-fold and 0.1 ml of the bacterial suspension was injected directly into drug-containing vials for smooth-colony variants. Because of the slower growth rate of the rough variant, the drug-containing vials were injected with 0.1 ml of the preculture without the dilution step. This permitted interpretation of results within 4 days for both smooth and rough variants of M. avium. The combined drug-inhibitor action was assessed by calculating x/y quotients as follows. x was the GI obtained with the combination of drug-inhibitor by using the BACTEC instrument, whereas y was the lowest GI obtained at the same time with the drug or the inhibitor used alone. For a two-drug combination, an x/y value of 1 indicated that there was no interaction between the two, a quotient of <0.5 indicated enhanced drug action, whereas an x/y quotient of >2 indicated the presence of antagonism between the drug and the inhibitor. Enhanced drug action was further verified by enumerating the CFU per milliliter at time zero and at the end of the experiment.

Drugs and inhibitors. The inhibitors used were avidin, bacitracin, 6-fluoro-6-deoxy-D-glucose, 2-deoxy-D-glucose, N-carbamyl-L-isoleucine, m-fluoro-phenylalanine, ethambutol, and cerulenin (Sigma Chemical Co., St. Louis, Mo.). Ciprofloxacin (Bayer Pharma, Puteaux, France), sparfloxacin (Rhône D.P.C. Europe, Antony, France), amikacin (Bristol, Paris, France), and clarithromycin (Abbott Laboratories, North Chicago, Ill.) were kindly provided by their manufacturers. Stock solutions of drugs and inhibitors were dissolved in distilled water and were sterilized by filtration through a 0.22 μm-pore-size filter prior to their addition to the vials. In the case of cerulenin, stock solutions were made by dissolving the drug in absolute ethanol. For clarithromycin, N-carbamyl-L-isoleucine, and m-fluoro-phenylalanine, methanol was used to dissolve the drugs, and in the case of avidin, phosphate-buffered saline was used. In these latter cases, appropriate controls included nontreated mycobacteria exposed to equivalent amounts of solvent to ensure that the inhibitory effects of the drugs were not due to the addition of solvent.

RESULTS

Isolation of rough variant of M. avium serovar 4. After the smooth-colony parent strain was allowed to grow at 37°C, using the previously described slant-tube procedure (5), the resulting pellicle was transferred to Middlebrook 7H9 agar plates and was streaked for isolation of colonies. The resulting rough colonies were then subcultured to Middlebrook 7H9 agar plates to ensure a stable rough-colony variant, which was subsequently used in the drug studies and radiolabeling experiments.

Results of BACTEC studies for inhibitory drugs. The inhibitory effects of the drugs were monitored for 4 days in the case of the smooth variants and 9 days in the case of the rough variants, because of their slower growth rate. Because cerulenin may lose up to 60% of its activity after 7 days at 27°C (28), a direct inoculum was used for the rough variant treated with cerulenin, and the culture was allowed to grow for 4 days. No growth inhibition of either the smooth or the rough variants was observed with 6-fluoro-6-deoxy-D-glucose (for concentrations of 100 and 500 μg/ml) or avidin (for concentrations ranging from 2 to 100 μg/ml). As summarized in Table 1, all other drugs were found to be inhibitory, but

TABLE 1. MICs of inhibitory drugs for smooth and rough variants of M. avium serovar 4

Inhibitory drug	MIC (μg/ml)	
	Smooth	Rough
Bacitracin	50	100
6-Fluoro-6-deoxy-D-glucose	>500	>500
2-Deoxy-D-glucose	1,000	1,000
N-Carbamyl-L-isoleucine	>100	100
Cerulenin	1	5
Avidin	>100	>100
DL-Ethionine	>100	>100
Ethambutol	5	5
m-Fluoro-phenylalanine	>100	100

results varied for the smooth and rough variants. A representative growth curve is given for cerulenin (Fig. 1).

In general, the drugs which are reported to affect glycosylation, i.e., 2-deoxy-D-glucose, bacitracin, and ethambutol, were inhibitory for both the smooth and the rough variants at equivalent concentrations (Table 1). For the
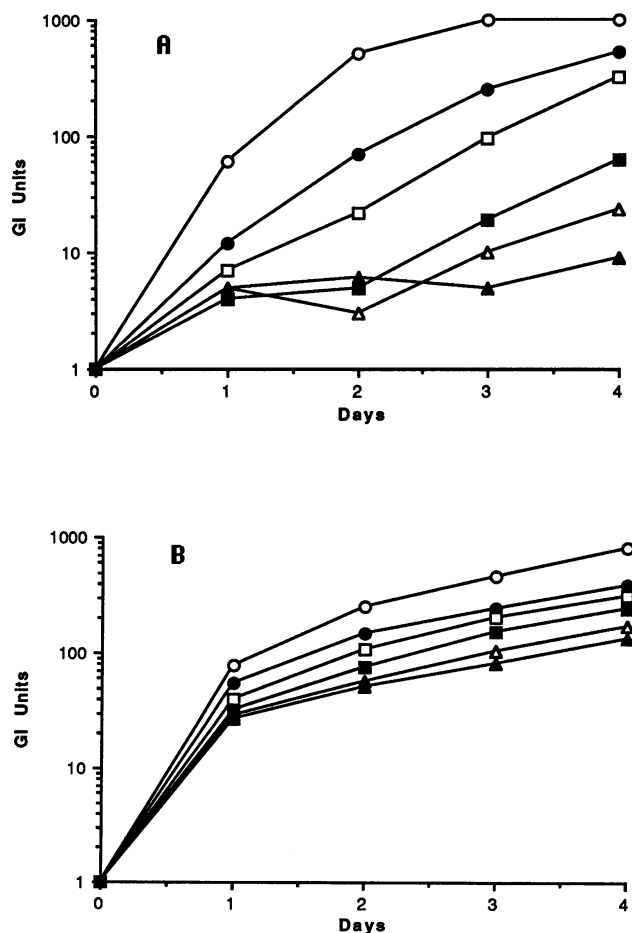

FIG. 1. Radiometric data showing the effects of cerulenin on M. avium serovar 4 smooth-colony (A) and rough-colony (B) variants. Growth is represented in terms of the GI values obtained with the BACTEC instrument measured for 4 days following exposure to drug. Concentrations of cerulenin were 0 μg (○), 0.25 μg (●), 0.5 μg (□), 1.0 μg (■), 2.0 μg (△), and 4.0 μg (▲) per ml.

TABLE 2. Radiometric data demonstrating combined antimicrobial-inhibitor activity against smooth and rough variants of M. avium serovar 4

Inhibitory drug	Ciprofloxacin (0.05)[b]		Sparfloxacin (0.05)		Clarithromycin (0.05)		Amikacin (0.5)	
	Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough
Bacitracin (5)[b]	–	–	–	–	–	–	+ (0.25)	–
N-Carbamyl-L-isoleucine	–	–	–	–	–	–	–	+ (0.45)
2-Deoxy-D-glucose (500)	–	–	+++ (0.05)	–	–	–	+ (0.17)	–
Cerulenin (0.5)	–	–	++ (0.09)	+ (0.32)	+ (0.36)	–	++++ (0.007)	++ (0.1)
m-Fluoro-phenylalanine (100)	–	–	–	–	–	–	+ (0.3)	+ (0.39)
Ethambutol (1)	–	–	+ (0.23)	–	–	–	+ (0.17)	+ (0.31)

[a] Values in parentheses are given as the quotient of x/y, where x is the GI obtained with the BACTEC instrument with the combination of inhibitor-drug, and y is the minimal GI values for drug or inhibitor used alone. An x/y of <0.5 indicates enhanced drug activity. For two-drug combinations, x/y values of >0.5 (–), ≤0.5 (+), ≤0.1 (++), ≤0.05 (+++), and ≤0.01 (++++) are given.

[b] Concentrations of drugs and inhibitors (in micrograms per milliliter).

drugs which are reported to affect peptide synthesis, i.e., N-carbamyl-L-isoleucine and m-fluoro-phenylalanine, growth inhibition was more effective against the rough variant than it was against the smooth variant. Likewise, the MICs of these two drugs indicated a similar response (Table 1). Cerulenin, which is reported to affect lipid synthesis, was effective against both smooth and rough variants but appeared to be more effective against the smooth variant at equivalent concentrations. This was also apparent with the MICs obtained for these variants (Table 1). The MICs of the antimicrobial agents did not vary for the smooth or rough variants and were 0.5, 0.5, 0.5, and 4 μg/ml for sparfloxacin, ciprofloxacin, clarithromycin, and amikacin, respectively.

BACTEC radiometric data for combined drug treatment. The results in terms of previously established x/y quotient calculations (36), obtained by using combined drug-inhibitor action for smooth- and rough-colony-type variants of M. avium, are summarized in Table 2. Typical radiometric data with some of the combinations and parallel controls are illustrated in Fig. 2 and 3. It must be emphasized here that all the drug or inhibitor concentrations selected for combined activity were chosen on the basis of MIC screening prior to the combined drug activity assessment. In this way, only sublethal concentrations of the inhibitors, along with subinhibitory concentrations of the drugs, were retained for the experiments reported below.

A total of nine drug-inhibitor combinations were found to possess an enhanced effect on smooth variants of M. avium (Table 2). On the other hand, only five drug-inhibitor combinations showed an enhanced inhibitory effect on the rough variants. If the degree of enhancement was assessed on the basis of x/y quotients, it was clear that the overall enhancement was much higher for the smooth variants. The drug showing the most promising activity in combination with the inhibitors used was amikacin; this was followed by sparfloxacin and clarithromycin, whereas ciprofloxacin did not show enhanced activity with any of the inhibitors used.

If the results were interpreted in terms of the three classes of the inhibitors used, i.e., those inhibiting glycosylation or fatty acid or peptide biosynthesis, it could be concluded that inhibitors of glycosylation (bacitracin and 2-deoxy-D-glucose) were more effective against the smooth-colony variant of M. avium than they were against the rough-colony variant (Table 2; Fig. 2 and 3). Among the potential inhibitors of glycosylation, ethambutol was able to enhance the activity of amikacin in the rough variant, but to a lower extent than

in the smooth variant (x/y quotients of 0.31 compared with 0.17 for the rough and smooth variants, respectively). However, ethambutol did not enhance the activity of sparfloxacin against the rough variant of M. avium, whereas it was able to do so in the case of the smooth variant (x/y quotient, 0.23). N-Carbamyl-L-isoleucine, on the other hand, was more effective against the rough variant, and m-fluoro-phenylalanine was about equally effective against both the smooth and the rough variants (Table 2; Fig. 3B). Inhibition of fatty acid biosynthesis (by cerulenin) appeared to be effective against both the smooth and rough variants, although it resulted in a much higher degree of enhancement in the case of the smooth variant (Table 2; Fig. 2B and 3A).

Rough variant. It has been well established that the smooth-colony strains of M. avium possess oligosaccharides which are attached to a lipopeptide core to make the serovar-specific GPL antigens. In addition, previous studies have revealed that 2-deoxy-D-glucose (49) and bacitracin (50) inhibit the biosynthesis of the GPL. Therefore, it was not surprising that inhibitors of glycosylation, such as bacitracin, 2-deoxy-D-glucose, and ethambutol, were more effective in combination with antimicrobial agents. However, it was interesting that the rough variant was more affected by combined treatment with inhibitors of peptide synthesis than by treatment with inhibitors of glycosylation. In an effort to explain these results, lipids from the rough variant that lacked the ability to synthesize GPLs were extracted and examined.

Lipids from the rough variant of serovar 4 were extracted and developed by TLC alongside lipid extracted from the respective smooth-colony-forming parent strain. Plates were sprayed with orcinol-sulfuric acids, and GPLs were identified by their intense yellow-gold color (7) (Fig. 4). The most obvious observation in the lipids extracted from the rough variant was the complete lack of the characteristic yellow-gold spots associated with the GPL serovar-specific antigens of the M. avium complex (Fig. 4, lane A). Although spots with R_f values similar to those of the GPLs were apparent in lipids from the rough variant (Fig. 4, lane B), these did not stain the characteristic yellow-gold color when sprayed with the orcinol reagent. This observation is more apparent when viewed in color. In addition, the absence of GPL was also confirmed by HPLC analysis of [^{14}C]Phe-radiolabeled lipid extracted from the rough variant (see below).

Incorporation of radiolabeled phenylalanine into lipids of the rough variant. In previous studies, [^{14}C]Phe has been
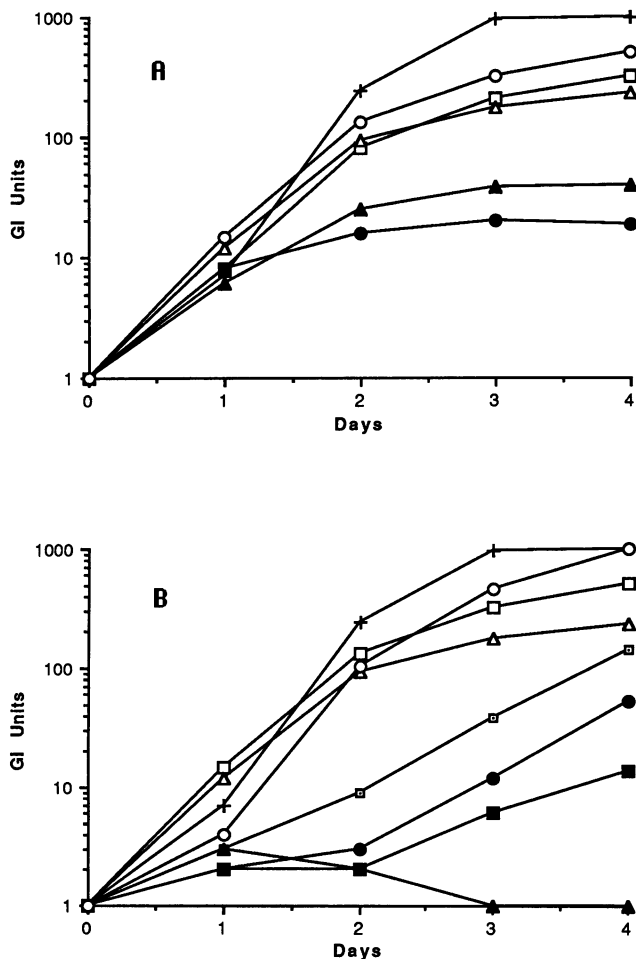
FIG. 2. (A) Radiometric data showing the effects of 2-deoxy-D-glucose used in combination with either amikacin or sparfloxacin on *M. avium* serovar 4 smooth-colony variants, in which case drugs or drug combinations are denoted as follows: 2-deoxy-D-glucose (□), amikacin (△), sparfloxacin (○), 2-deoxy-D-glucose–amikacin (▲), and 2-deoxy-D-glucose–sparfloxacin (●). (B) Radiometric data showing the effects of cerulenin used in combination with sparfloxacin, clarithromycin, or amikacin on *M. avium* serovar 4 smooth-colony variant. The drugs or drug combinations cerulenin (⊡), amikacin (△), sparfloxacin (□), clarithromycin (○), cerulenin-amikacin (▲), cerulenin-sparfloxacin (■), and cerulenin-clarithromycin (●) were tested. +, nontreated controls. Growth is represented in terms of the GI values obtained with the BACTEC instrument and measured for 4 days following exposure to drug. 2-Deoxy-D-glucose (500 μg/ml), amikacin (0.5 μg/ml), sparfloxacin (0.05 μg/ml), cerulenin (0.5 μg/ml), and clarithromycin (0.05 μg/ml) were used at the indicated concentrations.
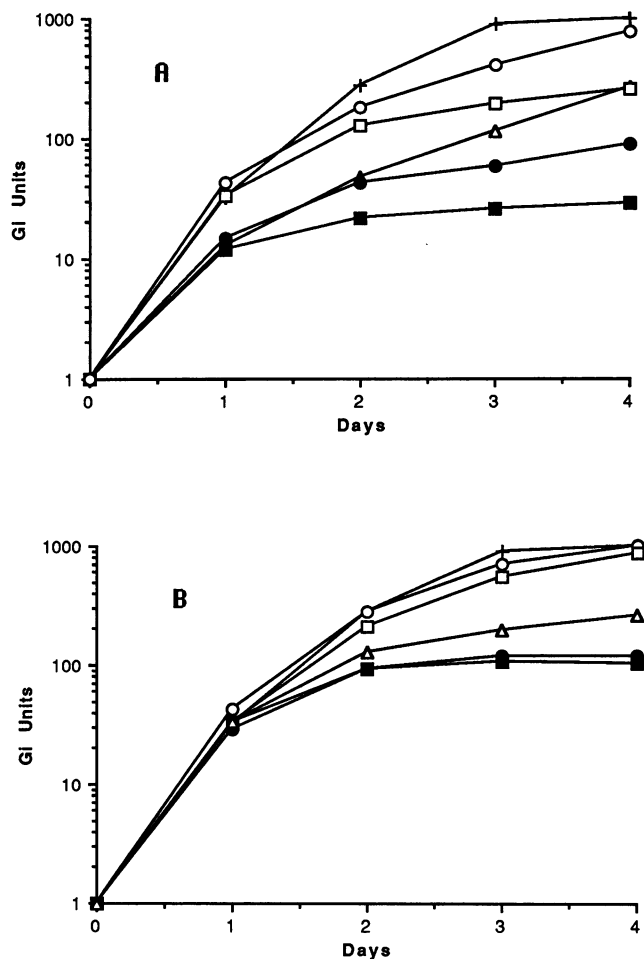
FIG. 3. (A) Radiometric data showing the effects of cerulenin used in combination with either sparfloxacin or amikacin on *M. avium* serovar 4 rough-colony variants, in which case the drugs or drug combinations cerulenin (△), amikacin (□), sparfloxacin (○), cerulenin-amikacin (■), and cerulenin-sparfloxacin (●) were tested. (B) Radiometric data showing the effects of *N*-carbamyl-L-isoleucine or *m*-fluoro-phenylalanine used in combination with amikacin on *M. avium* serovar 4 rough colony variant. The drugs or drug combinations *N*-carbamyl-L-isoleucine (○), *m*-fluoro-phenylalanine (□), amikacin (△), *N*-carbamyl-L-isoleucine–amikacin (●), and *m*-fluoro-phenylalanine–amikacin (■) were tested. +, nontreated controls. Growth is represented in terms of the GI values obtained with the BACTEC instrument and measured for 4 days following exposure to drug. Cerulenin (0.5 μg/ml), amikacin (0.5 μg/ml), sparfloxacin (0.05 μg/ml), *N*-carbamyl-L-isoleucine (100 μg/ml), and *m*-fluoro-phenylalanine (100 μg/ml) were used at the indicated concentrations.

used to internally radiolabel the GPLs and the apolar GPLs (18, 47). In the present study, [^{14}C]Phe was used to verify the absence of GPLs in rough variants and to identify any phenylalanine-containing lipopeptides that might be present as potential GPL precursors. Analysis of three separate experiments revealed that the average incorporation of [^{14}C] Phe into native lipid was 7.8×10^4 cpm/mg (range, 6.7×10^4 to 9.8×10^4 cpm/mg) for the rough variant of serovar 4.

[^{14}C]Phe-radiolabeled lipids from the rough variant were examined by HPLC to allow for a better separation of individual lipid components (Fig. 5). A major peak with a

retention time of 24 min was identified (Fig. 5A, peak 1), and a cluster of peaks with retention times ranging from 28 to 42 min was also observed (Fig. 5A), with a common peak having a retention time of 31 min (Fig. 5A, peak 2). Numerous HPLC runs (800 μg of lipid per run) were required to obtain sufficient quantities of peaks 1 and 2 for further analysis. It was determined that peaks 1 and 2 made up approximately 12 and 7% of the total lipid, respectively, and incorporation of ^{14}C was 1.8×10^5 cpm/mg of lipid for peak 1 and 8.1×10^5 cpm/mg of lipid for peak 2. The HPLC patterns of purified peaks 1 and 2 are given in Fig. 5B and
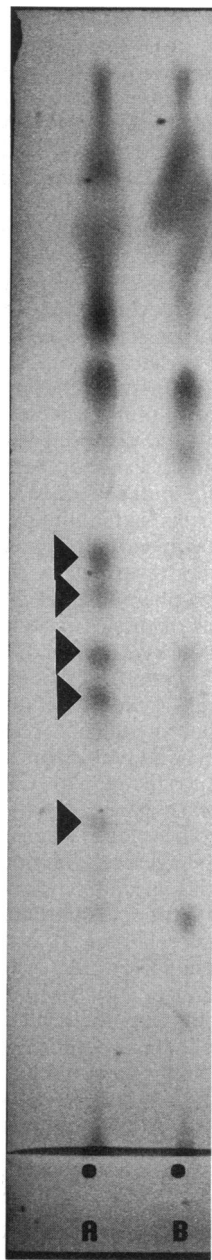
FIG. 4. TLC plate containing lipid extracted from smooth-colony (lane A) and rough-colony (lane B) variants of *M. avium* serovar 4. The plate was developed in solvent A, and GPLs were detected with orcinol-sulfuric acid reagent. GPLs are indicated by arrowheads.

FIG. 5. HPLC pattern of [^{14}C]Phe-radiolabeled native lipid (A) and purified [^{14}C]Phe-radiolabeled peak 1 (B) and peak 2 (C) of *M. avium* serovar 4 rough variant. Lipid resulting from deacylation procedure is given as a dashed line (C). Column conditions are given in the text.

5C, respectively, along with that of native lipid from serovar 4 (Fig. 5A).

Purified samples of peaks 1 and 2 were subjected to the deacylation procedure described by Brennan and Goren (7) and were reexamined by HPLC. It was determined that peak 1 is stable to the deacylation procedure, and analysis of its deacylation product produced an identical HPLC pattern with the same retention time (24 min) as that of the native sample (Fig. 5B). However, deacylation of peak 2 resulted in a product that had multiple peaks with greater retention times, suggesting the removal of acyl groups (Fig. 5C).
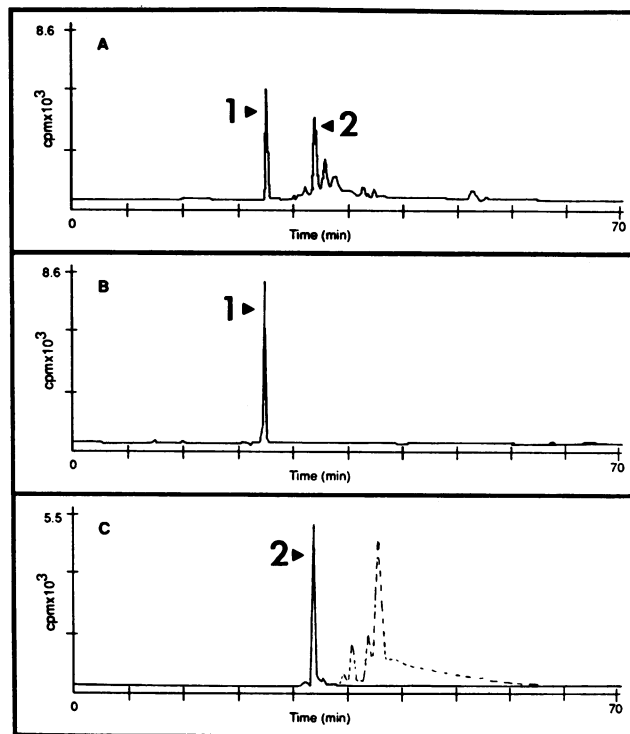
Chemical analysis of peaks 1 and 2. Samples of peaks 1 and 2 from serovar 4 were hydrolyzed for 72 h and were examined for the presence of amino acids by cochromatography on cellulose TLC with known standards and reaction with ninhydrin. The presence of [^{14}C]Phe was confirmed in each sample by cochromatography with a known sample of phenylalanine (solvent C; TLC data not shown) and subsequent removal of sample and assay in scintillation spectrophotometer (18, 49). Isoleucine (Ile) and alanine (Ala) were also identified in peak 1, and threonine (Thr), Ala, and alaninol (Alol) were identified in peak 2. Quantitation of amino acids following 72 h of hydrolysis revealed a molar ratio of 1.48:0.72:1.03 (Phe:Ile:Ala) for peak 1 and 2.76:4.42:3.98 (Phe:Thr:Ala) for peak 2. A 72-h hydrolysis was chosen because it has been demonstrated that it gives a more valid approximation of the amount of Phe in GPLs (7). Peak 1 was also hydrolyzed for 16 h to verify the absence of Thr because Thr can decompose during prolonged hydrolysis with 6 N HCl (15). No Thr in peak 1 was detected in 16-h hydrolysates either by cellulose TLC or with an amino acid analyzer. The ratio of Phe:Ile:Ala for peak 1 was found to be 1.23:0.71:1.25 in the 16-h hydrolysis. Carbohydrate analysis by the phenol-sulfuric acid procedure revealed that peak 1 had no carbohydrate and peak 2 contained 8.4% carbohydrate. In addition, analysis by ^{1}H nuclear magnetic resonance further confirmed the presence of phenylalanine and the absence of glycosyl residues in peak 1 and also confirmed the presence of OCH$_3$ groups in peak 2, which would explain the deacylation results (Fig. 5C) (6a).

Incorporation of [^{14}C]Ile. The rough variant of serovar 4 was cultured in the presence of [^{14}C]Ile to allow for internal
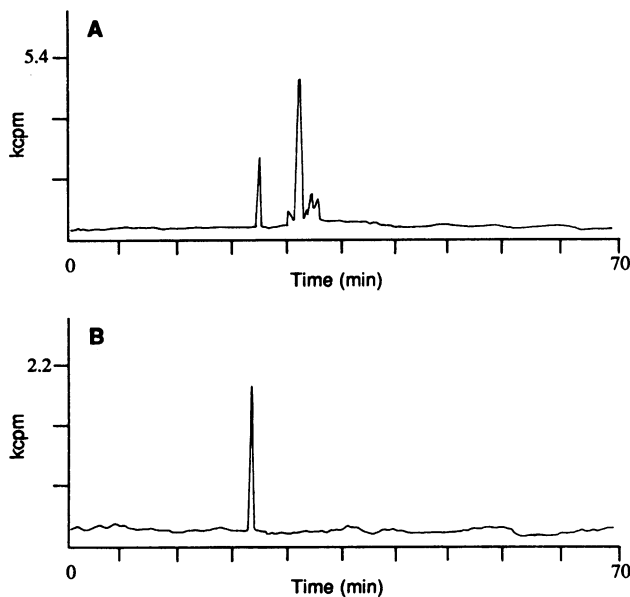
FIG. 6. HPLC pattern of [^{14}C]Phe-radiolabeled native lipid (A) and [^{14}C]Ile-radiolabeled native lipid (B) from *M. avium* serovar 4 rough variant. Column conditions are given in the text.

radiolabeling of peak 1 and any other isoleucine-containing components. Analysis of the native radiolabeled lipid extracted from the rough variant indicated that only one predominant component contained [^{14}C]Ile (Fig. 6). This component cochromatographed with purified samples of peak 1, and analysis of the hydrolysis product by the procedures described above confirmed the presence of [^{14}C]Ile.

DISCUSSION

The clinical significance of *M. avium* infections in human immunodeficiency virus-infected individuals, along with the reported resistance of these organisms to numerous antimicrobial agents, has resulted in a therapeutic problem for physicians. This problem has gained recent attention in the form of a National Institutes of Health workshop (23) and an Institut Pasteur forum (31) designed to identify and discuss high-priority areas important in the understanding of these significant opportunistic pathogens and in developing effective strategies for drug therapy. It has been suggested previously that multiple-drug resistance for *M. avium* cannot be explained by genetic factors or membrane-associated selective permeability but, instead, can be explained by the mycobacterial cell envelope architecture, which might act as a barrier for the exclusion of some drugs (10, 33). This suggestion has been further strengthened by studies in which it was determined that the combination of *m*-fluoro-phenyl-alanine, an inhibitor of mycoside-C biosynthesis (11), and ethambutol, an inhibitor of arabinogalactan biosynthesis (42), results in an enhancement of *M. avium* susceptibility (36). Subsequently, it was reported that the attachment of a palmitic acid side chain to isoniazid renders it growth inhibitory against *M. avium* and the use of this derivative along with *m*-fluoro-phenylalanine results in a bactericidal effect (34).

In a continuing effort to develop potential effective drug therapy for *M. avium* using the strategies described above,

we report here the use of drugs which have the potential to affect the biosynthesis of GPLs, a major component of the *M. avium* cell envelope (6). The results reported here indicate that inhibitors of glycosylation are effective for both smooth- and rough-colony variants of *M. avium*. Because the smooth-colony variant synthesizes the glycosylated serovar-specific GPLs (25, 47), it is not surprising that drugs such as 2-deoxy-D-glucose and bacitracin are effective, particularly since their inhibitory effects on GPL biosynthesis have been reported previously (49, 50). The fact that these drugs were inhibitory for the rough variant suggests that they have similar modes of action, except that the rough variant does not synthesize the complete GPL (5, 40). However, the rough variant can apparently synthesize a partially glycosylated lipopeptide that may be similar to the apolar GPL (3, 48). The inhibitory nature of these drugs may also suggest a glycosylated component(s) that is shared by both the rough and smooth variants, perhaps as a part of the reported polysaccharide outer layer (37).

With regard to the drugs that inhibit peptide synthesis, it is interesting that the rough variant appears to be more susceptible than the smooth variant to their action. As discussed above, *m*-fluoro-phenylalanine has been reported to inhibit the biosynthesis of the C-mycosides, meaning that it would inhibit not only the synthesis of GPL but the synthesis of the apolar GPL as well. Analysis of the rough variant indicates that although it does not synthesize the fully glycosylated GPL, it does synthesize phenylalanine-containing lipopeptides that may be precursors in GPL biosynthesis and that are similar to the apolar GPL (peaks 1 and 2 described in this report).

Cerulenin appears to be effective against both smooth and rough variants, which suggests a common fatty acid-containing component which is susceptible to that drug. It is known that cerulenin inhibits fatty acid synthetase (26, 27), but cell envelope-related events resulting from that inhibition in *M. avium* have not been elucidated. McCarthy (24) has reported that cerulenin inhibition of *M. avium* is reversible by octanoate, suggesting that octanoate may be critical to cell division. It is plausible that cerulenin might interfere with the synthesis of cell envelope constituents such as the GPLs, which contain a fatty acyl moiety; however, additional studies will be necessary to define this mechanism further.

A general assessment of the combined treatment indicates that drugs reported to be inhibitory for glycosylation are more effective in inhibiting the growth of the smooth variant when used in combination with antimicrobial agents than they are when used to treat the rough variant. Alternatively, drugs which are reported to be inhibitory for peptide synthesis are more effective in combined therapy against the rough variant. Lastly, ethambutol and cerulenin appear to be effective when used in combination against either the smooth or the rough variant.

Results regarding drugs that inhibit glycosylation suggest that glycosylated components are more abundant or contribute more to cell envelope integrity in the case of the smooth variant than in the case of the rough variant. This would explain the synergistic effect observed with that group of inhibitory drugs and the antimicrobial agents sparfloxacin, clarithromycin, and amikacin; these antimicrobial agents must penetrate the cell envelope to be effective. This is reasonable considering the fact that several important barriers of the mycobacterial cell wall are potentially affected: peptidoglycan, arabinogalactan, GPL, and the outer polysaccharide layer. Although no comparative chemical analysis has been reported for the cell envelope of smooth and

rough variants, it is known that the smooth variant possesses the fully glycosylated GPL components (25, 47, 49), whereas the rough variant does not (5; this study). This might be at least a partial explanation for the variation observed in the present investigation.

With regard to the results obtained with the drugs that affect peptide synthesis, it appears that lipopeptides or related components may be more abundant (or important to overall cell envelope integrity) in the rough variant than in the smooth variant. This would be one explanation for the synergistic effect of *N*-carbamyl-L-isoleucine and *m*-fluoro-phenylalanine observed with the antimicrobial agents sparfloxacin, clarithromycin, and amikacin. Again, no comparative chemical analysis has been reported for rough and smooth variants; however, as described in this report and previously (48), the rough variant does contain a substantial amount of lipopeptides which have amino acids (i.e., phenylalanine and isoleucine) that would suggest its susceptibility to those inhibitory drugs. Preliminary evidence has already demonstrated that *M. avium* is susceptible to *m*-fluoro-phenylalanine by virtue of its ability to inhibit C-mycoside (GPL) biosynthesis (11, 34, 36). If the phenylalanine-containing lipopeptides identified in the present investigation are precursors in GPL biosynthesis, then it is not unreasonable to expect that *m*-fluoro-phenylalanine would inhibit their biosynthesis as well and be effective against the rough variant. As reported here, two major lipopeptide components exist in the rough variant in substantial quantities (12 and 7% of total extractable lipid for peak 1 and peak 2, respectively).

In this report, we elaborated on two peptide-containing lipids (peaks 1 and 2) identified in a rough variant of the *M. avium* complex. These components were first described by us at the First International Conference on the Pathogenesis of Mycobacterial Infections, 1990 (30), and have been tentatively identified in at least two other *M. avium* rough variants (30, 48). Because the lipopeptides contain key amino acids previously found in the peptide moiety of the GPLs, it is possible that they may be associated with the biosynthesis of GPLs and, therefore, may represent potential targets for combined drug therapy. The presence of isoleucine in the lipid from peak 1 suggests its susceptibility to *N*-carbamyl-L-isoleucine, but the absence of threonine and alaninol makes it more difficult to explain the lipopeptide's direct role in GPL biosynthesis. This component, however, is identical (as determined by cochromatographic analysis by HPLC; data not shown) to one previously reported (49) to increase concomitantly when GPL biosynthesis is inhibited by 2-deoxy-D-glucose. It is interesting that a phenylalanine-isoleucine-alanine-containing lipopeptide was previously isolated from another species of mycobacteria, *M. johnei* (*M. paratuberculosis*), and was given the following proposed structure (22): $CH_3-(CH_2)_{20}-D-Phe-L-Ile-L-Ile-L-Phe-L-Ala-OCH_3$. Although the ratio of Ala:Ile: Phe determined for peak 1 does not conform to that of the lipopeptide identified in *M. johnei*, it is apparently the only other such lipopeptide reported in mycobacteria. We are attempting to purify sufficient quantities of the compounds that made up peaks 1 and 2 for a more detailed structural analysis. It is also interesting that when [^{14}C]Ile was used to • internally radiolabel the lipids in the rough variants, peak 1 was the only major component identified. Although attempts were not made to quantitate specific differences in the amounts of the lipopeptides in the smooth versus the rough variants, it is most likely that these lipopeptides make up a

far greater proportion of the lipid in the rough than in the smooth variants.

Whether or not these phenylalanine-containing lipopeptides participate in GPL biosynthesis will have to be determined by further studies. Because of the presence of phenylalanine, alanine, threonine, alaninol, and carbohydrate, it is more likely that the lipid associated with peak 2 is involved in GPL biosynthesis. However, conclusive evidence for the participation of peaks 1 and 2 in GPL biosynthesis will have to await a more detailed structural analysis and assay in the cell-free system that we have recently reported (29). Those studies are in progress.

Ethambutol has been reported to inhibit arabinogalactan biosynthesis in *Mycobacterium smegmatis* (42); therefore, it is likely that a similar mechanism took place in the combined therapy with the smooth and rough variants in our study. Although a comparative chemical analysis with regard to arabinogalactan has not been reported for smooth and rough variants of *M. avium*, the drug is effective in enhancing combined drug treatment of *M. avium* in vitro (36) and intracellularly within a J-774 macrophage cell line (38) as well as human and murine macrophages (39).

Lastly, the results with cerulenin indicate that inhibition of lipid biosynthesis increases the susceptibilities of both smooth and rough variants to combined antimicrobial treatment. Although this may be due to an effect on cell membrane integrity, that rationale does not completely explain the various susceptibility patterns of the smooth versus the rough variants. The results may suggest that a common group of lipid-containing components important in cell envelope integrity exists in the smooth and rough variants, but to various degrees. This may be further evidence substantiating our suggestions regarding the importance of the phenylalanine-containing lipopeptides in cell envelope integrity. Because cerulenin is an inhibitor of fatty acid synthetase (26), it is very likely that those lipid-containing components are also affected by treatment with cerulenin.

ACKNOWLEDGMENTS

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