ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1990, p. 759–764
0066-4804/90/050759-06$02.00/0

Vol. 34, No. 5

Enhancement of Drug Susceptibility of *Mycobacterium avium* by Inhibitors of Cell Envelope Synthesis

NALIN RASTOGI,* KHYE SENG GOH, AND HUGO L. DAVID

Unité de la Tuberculose et des Mycobactéries, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France

Received 6 November 1989/Accepted 7 February 1990

Treatment of infections caused by *Mycobacterium avium* complex bacteria still remains a challenge since these organisms are resistant to a majority of antituberculous drugs. *M. avium* is very often linked with acquired immune deficiency syndrome-associated opportunistic infections. We earlier suggested that one of the strategies for circumventing multiple-drug resistance might be the enhancement of *M. avium* drug susceptibility by inhibiting the synthesis of the outermost layer of its envelope, which appears to act as an exclusionary barrier for drugs. In this investigation, we have examined this strategy by simultaneously using drugs and the following inhibitors of the *M. avium* cell envelope: *m*-fluoro-phenylalanine (an inhibitor of mycoside-C biosynthesis), DL-norleucine (an inhibitor of transmethylation reactions), ethambutol (an inhibitor of arabinogalactan synthesis), EDTA (a divalent-ion chelator), and colistin (an inducer of membrane flux of divalent cations). All the drugs were used in concentrations which were low enough for a possible medical application to be foreseen. This approach, tested on seven strains of the *M. avium* complex, showed that both *m*-fluoro-phenylalanine and ethambutol were interesting candidates because they caused significant enhancement of *M. avium* drug susceptibility.

Mycobacterium avium, an opportunistic human pathogen, is a multiple-drug-resistant bacterium. Although successful therapy of *M. avium*-infected patients has long been a dilemma (7), the current acquired immune deficiency syndrome pandemic has unexpectedly increased the incidence of new cases, since *M. avium* is one of those opportunists which have found a privileged niche in immunosuppressed patients (26). Before the current coordinated research efforts to tackle this problem were started, we proposed that multiple-drug resistance of *M. avium* could not be satisfactorily explained by genetic factors (plasmids, mutation effects) or membrane-associated selective permeability and suggested that bacterial cell envelope architecture acted as a barrier for excluding some of the drugs (1, 14, 25).

From 1981 onward, this proposition was strengthened by our later studies, which showed that the mycobacteria were surrounded by an outer wall layer (OL) (6, 14); that drugs known to intercalate between bilayers (4) also disrupted mycobacterial OL and caused a Mg^{2+} ion flux (17); that *M. avium* spheroplasts were more drug susceptible than intact bacilli (6, 14); and that lipophilic drugs, which were better solubilized within the amphipathic substances of the OL, were also more active against bacteria (2), whereas a hydrophilic drug like isoniazid had increased activity against *M. avium* if it contained a lipophilic side chain (16).

We have also reported the inhibition of mycoside-C biosynthesis by *m*-fluoro-phenylalanine (FL-PHE) (5) and the inhibition of transmethylation in outer wall lipids by D-norleucine (3), both of which also caused alterations in the *M. avium* cell envelope.

The aim of this study was to investigate whether inhibition of *M. avium* cell envelope synthesis by various inhibitors could enhance the drug susceptibility of these bacilli. We included ethambutol (EMB) as one of the inhibitors in this study following a report that indicated that it increased the drug susceptibility of *M. avium* (9); EMB was also reported to inhibit both the transfer of mycolic acids into the cell wall (20) and the synthesis of mycobacterial arabinogalactan in *Mycobacterium smegmatis* (21). EDTA was also studied as an inhibitor to assess whether the chelation of Mg^{2+} in the outer layer could alter the wall enough to enhance bacterial drug susceptibility.

Some of the drug susceptibility data presented here, along with electron microscopy (EM) evidence, indicate that inhibition of envelope synthesis may be one of the most promising strategies for overcoming the drug resistance of *M. avium*.

MATERIALS AND METHODS

Bacteria and growth. Seven strains of *M. avium* complex (five laboratory-maintained strains, including the type strain ATCC 15769, and two recent clinical isolates) used in this investigation were from our own culture collection (see Table 2). The bacteria were grown in complete 7H9 medium (Difco Laboratories, Detroit, Mich.) containing 0.05% (vol/vol) Tween 80 to an optical density of 0.15 (measured at 650 nm with a Coleman Junior II spectrophotometer), which corresponded to about 10^8 viable counts per ml.

Radiometric drug susceptibility testing. The growth of *M. avium* was monitored radiometrically by using BACTEC 460-TB apparatus (Becton Dickinson, Towson, Md.). By this method, bacterial growth in a confined atmosphere with ^{14}C-labeled palmitate in Middlebrook 7H12a broth is measured as a function of the release of ^{14}C-labeled CO_2. The CO_2 in turn is captured by a detector, and growth is then expressed as a numerical value, called growth index (GI), which ranges from 1 to 999. The method as used by us (15) was as follows.

A vial containing 4 ml of broth was inoculated with 0.1 ml of a bacterial suspension (optical density of 0.15 at 650 nm, measured with a Coleman Junior II spectrophotometer), and once the GI reached 500, this primary culture was used as an inoculum for drug susceptibility testing. For this purpose, the bacterial suspension described above was diluted 10-

* Corresponding author.

fold, and 0.1 ml of it was then injected directly into drug-containing vials. The GIs of the drug-containing vials were compared with those of two control vials which were inoculated with 0.1 ml of 10- and 1,000-fold dilutions of the initial inoculum, respectively. The comparison of GIs in vials containing the drugs with that of the 10-fold-diluted control vial gave a direct indication of drug activity, whereas the comparison of GIs in drug-containing vials with that in the 1,000-fold-diluted control vial gave the drug susceptibility data by the 1% proportion method criterion. It should be pointed out here that because of the more rapid growth of atypical mycobacteria in the BACTEC system, the bacterial suspensions injected into the vials were 10-fold more diluted than recommended for *M. tuberculosis* (15).

Evaluation of combined drug action. The method used for evaluating the combined antibacterial effect of drugs with BACTEC radiorespirometry was applied as recommended by Hoffner et al. (9, 10). Briefly, the combined drug action is equal to X/Y, where X is the BACTEC GI obtained with a combination of wall inhibitor plus drug and Y is the lowest GI value obtained at the same time for the drugs or inhibitor used alone. An X/Y value of 1 indicated that there was no interaction between the two, an X/Y of <0.5 indicated an enhanced drug action, and an X/Y of >2.0 indicated the presence of antagonism between the drug and inhibitor.

In the present work, combined drug action was calculated 4 and 8 days after drug plus inhibitor were added to *M. avium* cultures. Also, counts of viable bacteria in the control vial at the time of inoculation and after 8 days of incubation were performed by plating serial dilutions on 7H11 agar medium and measuring CFU per milliliter after 21 days of incubation at 37°C. These control values were then compared with the CFU per milliliter values obtained for the vials containing various drugs and wall inhibitors after 8 days of incubation. CFU counts in vials containing both the wall inhibitors and drugs were determined only when the X/Y quotient was less than 0.5. Thus, the enhancement of drug action against *M. avium* was assessed not only by radiometric respirometry but also by conventional viable cell count determinations.

Drugs and inhibitors. In this study, the wall inhibitors used were D-norleucine, FL-PHE, EDTA, and colistin (or polymyxin E). All the inhibitors were purchased from Sigma Chemical Co., St. Louis, Mo.

The drugs used were isoniazid (INH), rifampin (RIF), (EMB), clofazimine, ofloxacin (OFLO), and ciprofloxacin (CIPRO). INH and RIF were purchased from Sigma. EMB from Laboratoire Lederle, Oullins, France; clofazimine from CIBA-GEIGY, Basel, Switzerland; OFLO from Roussel Uclaf, Paris, France; and CIPRO from Bayer Pharma, Puteaux, France, were kindly supplied by the manufacturers.

All of the stock solutions were prepared as indicated by the manufacturer and sterilized by filtration with a 0.45-μm-pore-size membrane filter.

Transmission EM. The action of those wall inhibitors which enhanced drug action was also verified at the level of mycobacterial cell envelope architecture. For this purpose, bacteria harvested during exponential growth phase were treated with the inhibitors for 24 h at 37°C and then fixed overnight at 4°C in a mixture of 1.25% (wt/vol) paraformaldehyde and 2.5% (wt/vol) glutaraldehyde containing 0.1% (wt/vol) ruthenium red (RR) in cacodylate buffer (0.1 M [pH 6.8] supplemented with 10 mM Ca^{2+} and 10 mM Mg^{2+}). The RR stain does not penetrate intact bacilli and has been used previously for showing the mycobacterial OL (14), which is otherwise not clearly visible by standard staining with uranyl acetate and lead citrate. The detailed transmission EM processing was performed as reported earlier (14), and thin sections were then mounted on Formvar-coated copper grids, double stained with lead citrate and uranyl acetate (to reveal ultrastructural details other than the OL), and observed with a Philips CM 12 EM.

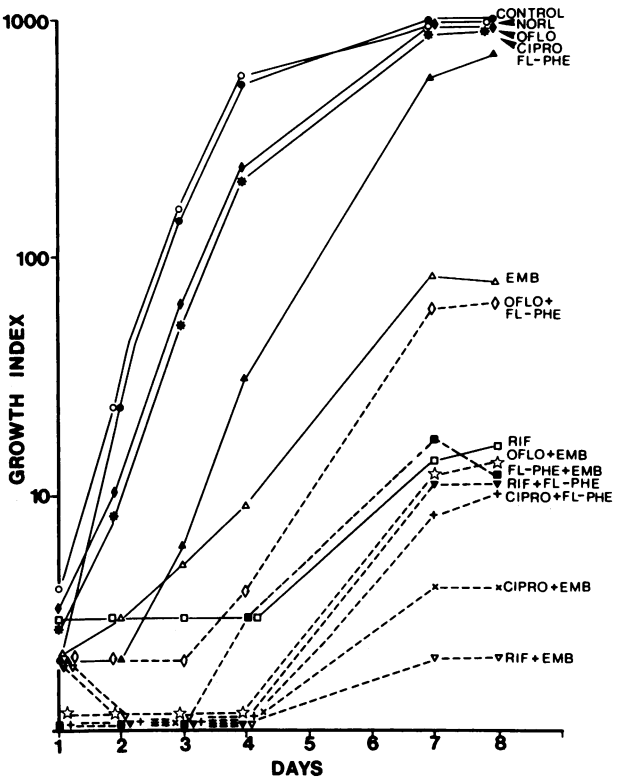


FIG. 1. Radiometric data showing the effects of various drugs, wall inhibitors, and drug-inhibitor combinations on the growth of *M. avium* ATCC 15769. Bacterial growth is represented in terms of BACTEC GI values measured for 8 days after the addition of drugs and inhibitors to 7H12a broth vials inoculated with 0.1 ml of 10-fold-diluted inoculum (see text for further details). NORL, D-Norleucine.

RESULTS

Radiometric results. The typical observations showing BACTEC GI values obtained by using various wall inhibitors, drugs, and drug-inhibitor combinations in the case of the type strain of *M. avium* (ATCC 15769) are shown in Fig. 1, and the combined drug action (calculated by using X/Y quotients at days 4 and 8) is summarized in Table 1. From these results, one can see that the inhibitors resulting in the highest enhancement of drug action were FL-PHE and EMB (EMB was used both as a wall inhibitor and as a drug). The drugs which showed the highest synergy with these wall inhibitors were RIF, OFLO, and CIPRO. None of the inhibitors enhanced INH action.

Neither Fig. 1 nor Table 1 shows our results with EDTA used as a divalent-ion chelator (used at 1 mM [372.5 μg/ml]) because EDTA used at 1 mM (although selected on the basis of a MIC determined by 7H9 broth dilution) was much too inhibitory for BACTEC radiorespirometry and thus did not give interpretable GI values for X/Y quotient calculations.

TABLE 1. Radiometric data showing anti-*M. avium* ATCC 15769 activity of various drugs and cell wall inhibitors used together

Inhibitor (μg/ml)	Drug (μg/ml)	*X*/*Y* quotient[a]	
		Day 4	Day 8
NORL (50)	INH (0.2)	0.90	1.00
	EMB (2.5)	0.90	1.02
	RIF (2.0)		0.87
	OFLO (1.0)	0.97	1.00
	CIPRO (1.0)	1.04	1.00
FL-PHE (50)	INH (0.2)	0.90	1.02
	EMB (2.5)	0.33	0.15
	RIF (2.0)		<0.06
	OFLO (1.0)	0.13	0.09
	CIPRO (1.0)	<0.03	0.02
EMB (2.5)	INH (0.2)	0.78	1.08
	COL (5.0)[b]	0.95	1.00
	RIF (2.0)		<0.06
	OFLO (1.0)	0.11	0.14
	CIPRO (1.0)	<0.10	0.05
COL (5.0)	INH (0.2)	0.95	1.00
	RIF (2.0)		1.12
	OFLO (1.0)	0.96	1.00
	CIPRO (1.0)	1.03	1.00

[a] *X* is the BACTEC GI obtained with the combination of wall inhibitor and drug; *Y* is the minimal GI value for drug or inhibitor used alone. An *X*/*Y* of <0.5 indicated enhanced drug action, an *X*/*Y* of 1 indicated a lack of interaction between the drug and inhibitor, and an *X*/*Y* of >2.0 indicated antagonism between the two.

[b] COL, Colistin.

Similarly, our results with clofazimine were not interpretable, since this drug, even when used at its critical concentration of 1 μg/ml, was much too active radiometrically for any combined drug action to be assessed with our experimental system. Consequently, in all of the cases in which radiometric data could not be interpreted clearly (as in combinations with 1 mM EDTA or 1 μg of clofazimine per ml) or in which there was a clear lack of enhanced drug action (as in combinations with 5 μg of colistin or 50 μg of D-norleucin per ml), the next step, determining viable cell counts, was omitted.

Enhancement of drug action as assessed by viable cell counts. The bacterial viable cell counts in the control BACTEC vials were performed both at the time of inoculation and after 8 days of incubation at 37°C. The bacterial cell counts at the time of inoculation of the vials were given a value of 1, and the growth in control as well as in drug- and inhibitor-containing vials was then compared after 8 days of incubation at 37°C by plating the bacterial suspensions from the vials onto 7H11 agar medium. Also, all the inhibitor-drug combinations showing an enhanced action (*X*/*Y* < 0.5; Table 1) were titrated for viable cell counts. Typical results obtained in the case of the type strain of *M. avium* (ATCC 15769) are summarized in Fig. 2. Anti-*M. avium* activity of all of the drugs except INH was enhanced by FL-PHE and EMB. These viable cell count data are extremely interesting since they not only showed enhanced action of the various drug-inhibitor combinations in the BACTEC system but also confirmed that some of the combinations tested were clearly bactericidal against *M. avium*.

Assessment of drug-wall inhibitor combinations against various strains of *M. avium* complex. Table 2 summarizes our results with a total of seven *M. avium* complex strains, including the type strain ATCC 15769. For drug-wall inhibitor combinations, the criteria for evaluating an enhanced action with BACTEC radiorespirometry were the same as those defined above. As can be seen from our results, none of the inhibitors enhanced the action of INH used at 0.2 μg/ml. When EMB was used as an inhibitor, it enhanced the activity of RIF, OFLO, and CIPRO in all of the strains tested. FL-PHE, on the other hand, caused enhancement of all of the above-named drugs in only two of seven strains and of RIF alone in six of seven strains.

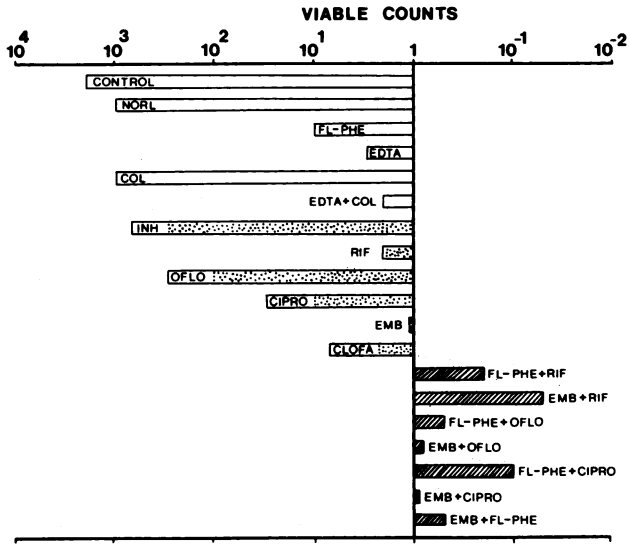
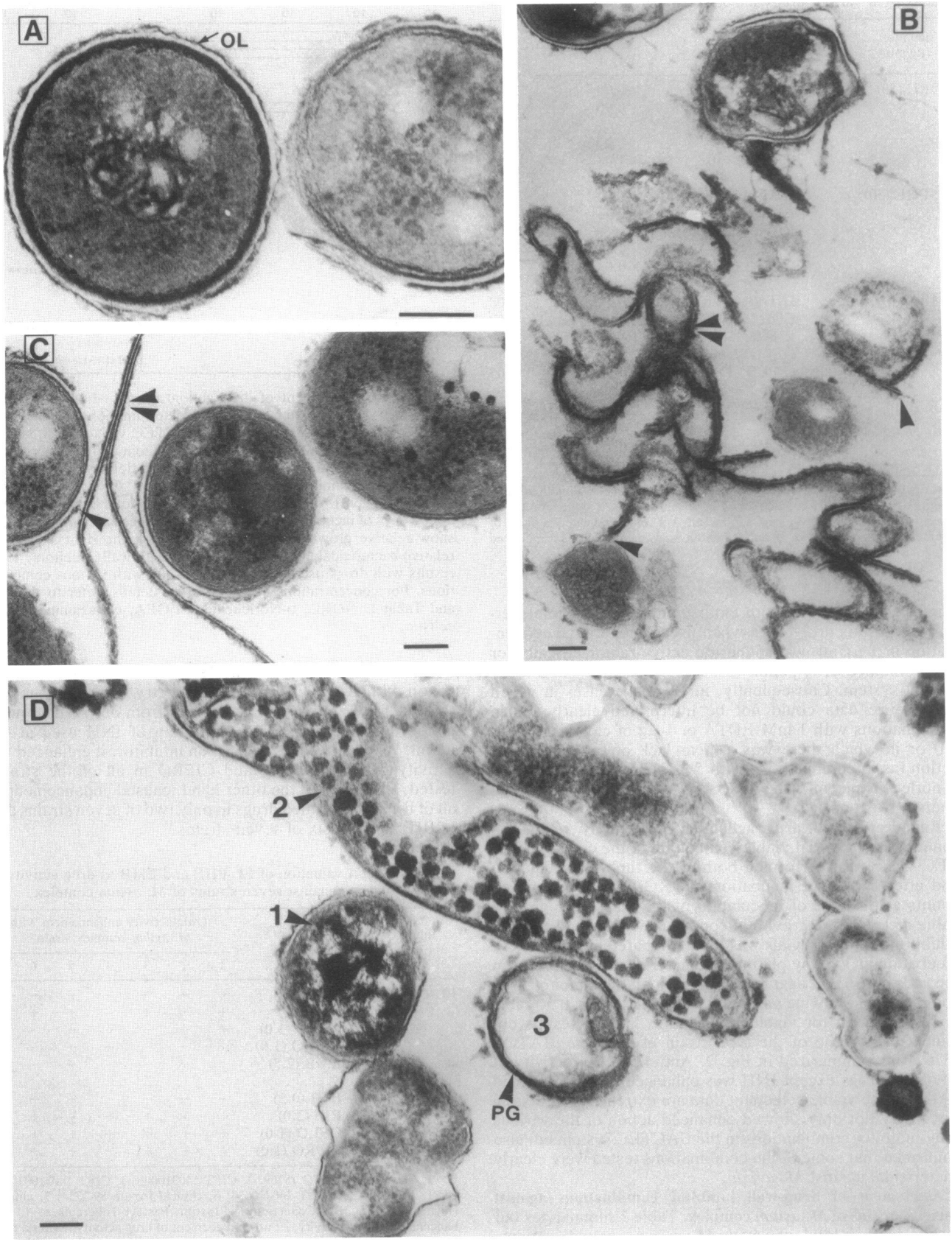


FIG. 2. Enhancement of drug action against *M. avium* ATCC 15769 measured by viable cell count determinations. Relative *M. avium* viable cell counts in various BACTEC vials after 8 days of incubation at 37°C are shown. The vials containing various wall inhibitors, drugs, and drug-inhibitor combinations were inoculated with the same number of bacilli (about 3×10^4 CFU; given a value of 1 in the figure). The control bar shows the total bacterial growth after 8 days of incubation at 37°C, whereas other bars on the left side show relative growth inhibition and bars on the right side show relative bactericidal effects. ☐, Results with wall inhibitors; ▩, results with drugs used alone; ▨, results with various combinations. For concentrations used and other details, refer to the text and Table 1. NORL, D-Norleucine; CLOFA, clofazamine; COL, colistin.

TABLE 2. Evaluation of FL-PHE and EMB as drug activity enhancers against seven strains of *M. avium* complex

Inhibitor (μg/ml)	Drug (μg/ml)	Drug activity enhancement with *M. avium* complex strain[a]:						
		1	2	3	4	5	6	7
FL-PHE (50)	INH (0.2)	−	−	−	−	−	−	−
	RIF (2.0)	+	+	+	−	+	+	+
	OFLO (1.0)	+	−	−	−	+	−	−
	CIPRO (1.0)	+	−	−	−	+	−	−
	EMB (2.5)	+	−	−	−	−	−	−
EMB (2.5)	INH (0.2)	−	−	−	−	−	−	−
	RIF (2.0)	+	+	+	+	+	+	+
	OFLO (1.0)	+	+	+	+	+	+	+
	CIPRO (1.0)	+	+	+	+	+	+	+

[a] 1, Type strain ATCC 15769; 2, CIPT 140310006; 3, CIPT 140310030; 4, CIPT 140310031; 5, CIPT 140310034; 6, clinical isolate 89-1734; 7, clinical isolate 89-1863. CIPT, Collection de l'Institut Pasteur-Tuberculose. +, Enhancement of drug activity; −, no enhancement of drug activity. Criteria used to evaluate the enhancement of drug action were the same as those defined in the text and in Table 1, footnote *a*.


A
OL
B
C
D
1
2
3
PG

EM. Since FL-PHE and EMB were the inhibitors which clearly enhanced drug action against *M. avium*, we also decided to investigate their action at the level of *M. avium* cell envelope architecture. For all ultrastructural investigations, we used the type strain ATCC 15769.

Our EM data obtained by double staining bacteria with RR (to show the OL) and with uranyl acetate plus lead citrate (to show EM details other than the OL) are illustrated in Fig. 3. Figure 3A shows an untreated control preparation; notice that the bacilli are intact, with a ribosome-rich cytoplasm, a DNA-rich nucleoplasm, a visibly asymmetric cytoplasmic membrane, a 10-nm-thick peptidoglycan (PG), and an equally thick electron transparent layer (ETL) which separates the PG from the RR-stained OL. The total wall thickness (comprising PG, ETL, and OL) in control bacilli was about 30 nm.

Although the effect of FL-PHE against *M. avium* has been described in detail earlier (5), a characteristic EM illustration showing the effect of 50 μg of FL-PHE per ml (24 h at 37°C) against the OL of *M. avium* is depicted in Fig. 3B. As can be seen from this electron micrograph, FL-PHE dismantles the bacterial OL, which is often lost in the surrounding medium, and reorganizes to form a structural bilayer. These newly formed bilayers remain stainable by the RR method. FL-PHE, however, does not cause bacterial lysis. These results agree with the proposed mechanisms of action of FL-PHE, which are the inhibition of mycoside-C biosynthesis along with the disorganization of the OL of *M. avium*, which is due to its action at the level of amphipathic substances responsible for the structural integrity of the OL (5, 6).

Our EM results with EMB are illustrated in Fig. 3C and D. Like FL-PHE, EMB appears to result in the disorganization of the bacterial OL (Fig. 3C), which may be released into the surrounding medium to form the characteristic bilayers (Fig. 3C), but it also causes the cytolysis of bacteria, the various steps of which are illustrated in Fig. 3D. In Fig. 3D, step 1 shows the plasmolysis of the bacilli, with the disappearance of ribosomes and appearance of symmetric cytoplasmic membranes. This is followed by aggregation and clearing of the bacterial cytoplasm (step 2) and, ultimately, formation of cell ghosts whose cell contents have leaked outside. The bacterial PG, however, remains, maintaining the ridgidity of the cell skeleton. These EM observations are in agreement with a recent report showing that EMB inhibits the synthesis of mycobacterial arabinogalactan (21), which may in turn cause the release of mycolic acid residues that normally esterify the arabinogalactan-mycolate, consequently releasing the bacterial OL, which is otherwise linked to the arabinogalactan-mycolate through lipid-lipid interactions in the ETL layer of the wall (6).

DISCUSSION

In the case of *M. avium*, exclusion at the level of the cell envelope has been proposed as the mechanism responsible for its multiple-drug resistance (1, 14). According to the *M. avium* wall model (6), the various amphipathic substances (observed in the OL by RR staining in transmission EM studies) are linked to the mycolic acid residues in arabinogalactan-mycolate by lipid-lipid interactions (the ETL observed with EM) and constitute a barrier to the efficient penetration of antimicrobial agents. It is not, however, a selectively permeable barrier, as the removal of the OLs results in enhanced permeation by a variety of substrates and drugs against *M. avium* (6, 14). We have proposed the term exclusion barrier to differentiate this barrier from the permeability barrier, since the term permeability denotes a specific transport-related phenomenon at the cytoplasmic-membrane level (1, 6, 14, 25).

In this investigation, we have clearly shown that inhibition of specific components in the *M. avium* cell envelope may be fruitful for developing new strategies in the chemotherapy of these infections. EMB was a better drug activity enhancer than was FL-PHE (Table 2).

We have also confirmed that the specific action of EMB at the level of transfer of mycolic acids (20) and inhibition of arabinogalactan (21) results in the disorganization of the *M. avium* OL, as evidenced by ultrastructural examinations (Fig. 3C). Also, cytolysis of the bacilli may result, but cytolysis causes the formation of bacterial ghosts without the dissolution of the bacterial PG (Fig. 3D); the latter is unlike the effect of D-cycloserine, which acts specifically at the level of bacterial PG biosynthesis, resulting in bacterial lysis (13).

FL-PHE, on the other hand, inhibits mycoside-C biosynthesis in *M. avium* and the consequent disorganization of the amphipathic substances in the bacterial OL (5), which may in turn cause increased drug entry.

These differences in the sites of action of the two wall inhibitors may also explain why EMB turned out to be a wide-range drug enhancer compared with FL-PHE (Table 2). Indeed, strain to strain and physiological variations in the composition of surface amphipathic substances and their topography in the OL architecture were suggested earlier (5). The arabinogalactan and mycolic acid compositions, on the other hand, do not vary significantly among the strains of a given species. Accordingly, FL-PHE may not cause sufficient disorganization of the OL of *M. avium* in all strains.

The fact that there is no enhancement of INH action by EMB may be explained by their respective mechanisms of action. INH inhibits the synthesis of mycolic acids (22, 24), thus having a site close to that of EMB, which, by inhibiting the synthesis of arabinogalactan, also liberates wall mycolic acid residues forming the arabinogalactan-mycolate layer. Indeed, Kilburn and Takayama (11) reported the accumulation of free mycolic acids in *M. smegmatis* treated with EMB.

We have used the term enhanced drug action instead of synergy because our experimental approach does not use

FIG. 3. Ultrastructure of *M. avium* ATCC 15769 treated with FL-PHE and EMB. All preparations were double stained with RR to reveal the bacterial OL and with lead citrate and uranyl acetate to reveal ultrastructural details other than the OL. (A) Untreated control bacilli showing a ribosome-rich cytoplasm, asymmetric cytoplasmic membranes, a 10-nm-thick PG, and an equally thick ETL (corresponding to the arabinogalactan-mycolate in the cell wall skeleton [6]) which separated the PG from the RR-stained OL. Total wall thickness was about 30 nm. (B) FL-PHE-treated preparation showing the dismantling of the OL (single arrow), which was often released into the surrounding medium and reorganized to form a structural bilayer (double arrows). (C and D) EMB-treated *M. avium* bacilli. Bacteria in panel C show the same phenomenon of ultrastructural disorganization of the OL as that in the FL-PHE-treated cells in panel B. However, EMB also caused cytolysis of the bacteria, the various steps of which are illustrated in panel D. Shown are the beginning of plasmolysis of a bacillus with the disappearance of ribosomes and symmetric cytoplasmic membranes (step 1), aggregation and clearing of the bacterial cytoplasm (step 2), and formation of cell ghosts (step 3). Notice that the ghosts were formed through the disorganization of ETL and OL, but bacterial PG was not lysed. Bars, 100 nm.

established criteria to define drug synergy (12). Our observations are closer to those in studies in which increased drug activity resulting from use of a selective enzyme inhibitor (18) or a bacterial wall modifier (19, 23) have been reported. Although Hoffner et al. (9, 10) originally used the term synergy to denote anti-*M. avium* activity of a drug combination if X/Y was less than 0.5, we prefer to use the term enhanced drug action. It should be mentioned here that our model also took into account viable cell count determinations. Whatever the terminology may be, the latter confirmed that if a combination was highly active radiometrically (as defined in the text), then it was also clearly bactericidal by standard viable cell count determination.

The MIC/MBC ratio for EMB against most of the *M. avium* strains are between 1/8 and 1/4 (8). In the present investigation, the association of EMB with other drugs and inhibitors has considerably changed this value, in some cases to 1/2 to 1/1, suggesting a clear bactericidal action at concentrations achievable in patients (e.g., in the case of EMB plus RIF). Also, an enhancement in the antibacterial activity of new fluoroquinolones was observed.

Consequently, our data suggest that enhancing drug action against *M. avium* by inhibiting specific wall components may be a strategy of choice in the development of future chemotherapeutic protocols. This investigation also strengthens a recent proposal that EMB may be a key component in various drug associations to be attempted for a successful therapy of *M. avium* infections (9). The BACTEC radiometric method appears today to be the easiest way to screen various drug combinations. In addition, viable cell count data associated with the GI synergy quotient calculations may give the information necessary to interpret valuable MIC/MBC ratios. Further studies evaluating various drug combinations must be performed.

ACKNOWLEDGMENTS

We thank Sylviane Cadou for helping with embedding and ultratomy of EM preparations. We are grateful to Becton Dickinson, France, for a kind gift of 7H12a broth vials and for lending a BACTEC 460-TB apparatus.

LITERATURE CITED

1. **David, H. L.** 1981. Basis for the lack of drug-susceptibility of atypical mycobacteria. Rev. Infect. Dis. **3**:878–884.
2. **David, H. L., S. Clavel-Sérès, F. Clément, and K. S. Goh.** 1987. Uptake of selected antibacterial agents in *Mycobacterium avium*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **265**:385–392.
3. **David, H. L., S. Clavel-Sérès, F. Clément, A. Laszlo, and N. Rastogi.** 1989. Methionine as a methyl-group donor in the synthesis of *Mycobacterium avium* envelope lipids, and its inhibition by DL-ethionine, D-norleucine and DL-norleucine. Acta Leprologica (Geneva) **7**(Suppl. 1):77–80.
4. **David, H. L., and N. Rastogi.** 1985. Antibacterial action of colistin (polymyxin E) against *Mycobacterium aurum*. Antimicrob. Agents Chemother. **27**:701–707.
5. **David, H. L., N. Rastogi, S. Clavel-Sérès, and F. Clément.** 1988. Alterations in the outer wall architecture caused by the inhibition of mycoside C biosynthesis in *Mycobacterium avium*. Curr. Microbiol. **17**:61–68.
6. **David, H. L., N. Rastogi, S. Clavel-Sérès, F. Clément, and M.-F. Thorel.** 1987. Structure of the cell envelope of *Mycobacterium avium*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **264**:49–66.
7. **Etzkorn, E. T., S. Aldarondo, C. K. McAllister, J. Matthews, and A. J. Ognibene.** 1986. Medical therapy of *Mycobacterium avium-intracellulare* pulmonary disease. Am. Rev. Respir. Dis. **134**:442–445.
8. **Heifets, L. B., M. D. Iseman, and P. J. Lindholm-Levy.** 1986. Ethambutol MICs and MBCs for *Mycobacterium avium* complex and *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **30**:927–932.
9. **Hoffner, S. E., G. Källenius, A. E. Beezer, and S. B. Svenson.** 1989. Studies on the mechanisms of synergistic effects of ethambutol and other antibacterial drugs on *Mycobacterium avium* complex. Acta Leprologica (Geneva). **7**(Suppl. 1):195–199.
10. **Hoffner, S. E., S. B. Svenson, and G. Källenius.** 1987. Synergistic effects of antimycobacterial drug combinations on *Mycobacterium avium* complex determined radiometrically in liquid medium. Eur. J. Clin. Microbiol. **6**:530–535.
11. **Kilburn, J. O., and K. Takayama.** 1981. Effects of ethambutol on accumulation and secretion of trehalose mycolates and free mycolic acid in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **20**:401–404.
12. **Krogstad, D. J., and R. C. Moellering, Jr.** 1980. Combinations of antibiotics, mechanisms of interaction against bacteria, p. 298–341. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
13. **Rastogi, N., and H. L. David.** 1981. Ultrastructural and chemical studies on wall deficient forms, spheroplasts and membrane vesicles from *Mycobacterium aurum*. J. Gen. Microbiol. **124**: 71–79.
14. **Rastogi, N., C. Frehel, A. Ryter, H. Ohayon, M. Lesourd, and H. L. David.** 1981. Multiple drug resistance in *Mycobacterium avium*: is the wall architecture responsible for the exclusion of antimicrobial agents? Antimicrob. Agents Chemother. **20**:666–677.
15. **Rastogi, N., K. S. Goh, and H. L. David.** 1989. Drug susceptibility testing in tuberculosis: a comparison of the proportion methods using Löwenstein-Jensen, Middlebrook 7H10 and 7H11 agar media and a radiometric method. Res. Microbiol. **140**:405–417.
16. **Rastogi, N., B. Moreau, M.-L. Capmau, K. S. Goh, and H. L. David.** 1988. Antibacterial action of amphipathic derivatives of isoniazid against the *Mycobacterium avium* complex. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **268**:456–462.
17. **Rastogi, N., M.-C. Potar, J.-G. Henrotte, G. Franck, and H. L. David.** 1988. Further studies on colistin (polymyxin E)-induced cell leakage in mycobacteria: Mg^{++} efflux in *Mycobacterium avium* and its effect on drug-susceptibility. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **268**:251–258.
18. **Reading, C., and M. Cole.** 1977. Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **11**:852–857.
19. **Soper, T. S., and J. M. Manning.** 1976. Synergy in the antimicrobial action of penicillin and β-chloro-D-alanine in vitro. Antimicrob. Agents Chemother. **9**:347–349.
20. **Takayama, K., E. L. Armstrong, K. A. Kunugi, and J. O. Kilburn.** 1979. Inhibition by ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **16**:240–242.
21. **Takayama, K., and J. O. Kilburn.** 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **33**:1493–1499.
22. **Takayama, K., L. Wang, and H. L. David.** 1972. Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **2**:29–35.
23. **Vaara, M., P. Viljanen, S. Sukupolvi, and T. Vaara.** 1985. Does polymyxin B nonapeptide increase outer membrane permeability in antibiotic supersensitive enterobacterial mutants? FEMS Microbiol. Lett. **26**:289–294.
24. **Winder, F. G., and P. B. Collins.** 1970. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis*. J. Gen. Microbiol. **63**:41–48.
25. **Woodley, C. L., and H. L. David.** 1976. Effect of temperature on the rate of the transparent to opaque colony type transition in *Mycobacterium avium*. Antimicrob. Agents Chemother. **9**:113–119.
26. **Young, L. S., C. B. Inderlied, O. G. W. Berlin, and M. S. Gottlieb.** 1986. Mycobacterial infections in AIDS patients with an emphasis on the *M. avium* complex. Rev. Infect. Dis. **8**:1024–1033.