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Determination of In Vitro Susceptibility of *Mycobacterium tuberculosis* to Cephalosporins by Radiometric and Conventional Methods

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Among eight cephalosporins and cephamycins tested in preliminary in vitro screening against *Mycobacterium tuberculosis*, the most promising for further study was found to be ceforanide, followed by ceftizoxime, cephapirin, and cefotaxime. Moxalactam, cefoxitin, cefamandole, and cephalothin were found to be not active enough against *M. tuberculosis* to be considered for further in vitro studies. The antibacterial activity of various ceforanide concentrations was investigated by three methods: (i) the dynamics of radiometric readings (growth index) in 7H12 broth; (ii) the number of CFU in the same medium; and (iii) the proportion method on 7H11 agar plates. There was a good correlation among the results obtained with these three methods. The MIC for most strains ranged from 6.0 to 25.0 μg/ml. The BACTEC radiometric method is a reliable, rapid, and convenient method for preliminary screening and determination of the level of antibacterial activity of drugs not commonly used against *M. tuberculosis*.

The first attempts to study the activity of β-lactamase-resistant penicillins against *Mycobacterium tuberculosis* were made after it was ascertained that the resistance of *M. tuberculosis* to penicillin G was related to β-lactamase activity (1, 2). In a study by Lorian and Sabath (3), cloxacillin had a bactericidal effect in a concentration of 10 μg/ml on five of six tested strains. Misiek et al. (6) screened more than 600 semisynthetic cephalosporins against the H37Rv strain by defining the MIC by broth dilution methods (presumably by turbidity measurement) and concluded that potential antituberculosis cephalosporins should have pyridyl or aminomethylphenyl moieties in a side chain at the C-7 position. By using this approach, Sanders et al. (9) compared two cephalosporins having one or the other of these characteristics (cephapirin and ceforanide) with five having neither (cephalothin, cephalexin, cephaloglycin, cefazolin, and cephaloridine). Ceforanide and cephapirin, followed by cephalothin, were found to be the most active. Further progress in this field requires the screening of many drugs against a large number of clinical isolates which have different patterns of susceptibility to the standard antituberculosis drugs. One of the difficulties in such screening is that it is extremely labor intensive to plate the growing broth cultures at many time points to determine antibacterial effect. On the other hand, determining growth inhibition of *M. tuberculosis* only by measuring changes in the turbidity would not be reliable or sufficiently sensitive. The recent development of the radiometric method to detect mycobacterial growth has provided new opportunities for the in vitro screening of many drugs (including nonstable drugs). The radiometric method, by using advanced technology especially developed for mycobacteriology (BACTEC system 460-TB), has proven to be an effective tool in drug susceptibility testing (5, 7, 10, 11).

The main aim of this study was to determine whether this radiometric method is a reliable technique for rapid screening of different antimicrobial agents and to determine the level of their activity against the clinical isolates of *M. tuberculosis*.

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MATERIALS AND METHODS

Antimicrobial agents. The cephalosporins and cephamycins for this study were supplied by different manufacturers. Cephalothin sodium for injections (Keflin), cefamandole (Mandol), and moxalactam (Moxam) were obtained from Eli Lilly & Co., Indianapolis, Ind. Cefoxitin sodium (Mefoxin) was obtained from Merck Sharp & Dohme, West Point, Pa. Ceftizoxime sodium (Cefizox) is manufactured by Fujisawa SmithKline Corp., Osaka, Japan, and distributed by Smith Kline & French Laboratories, Philadelphia, Pa. Cefotaxime sodium (Claforan) was obtained from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J. Cephapirin (Cefadyl) and ceforanide (Precef) were supplied by Bristol Laboratories, Syracuse, N.Y. The drugs were reconstituted as specified by the manufacturers and then diluted to a concentration of 2,000 μg/ml in diluting fluid (DF), which consists of a 0.2% solution of bovine albumin fraction V (Sigma Chemical Co., St. Louis, Mo.) in 0.02% Tween 80. Drug solutions were distributed in small aliquots, kept frozen at −70°C, and used for preliminary screening. The addition of 0.1 ml of the solution to 2.0 ml of 7H12 broth in a vial produced a final concentration of 100 μg/ml in the medium. Twofold dilutions of the 2,000-μg/ml solution were made in DF and kept as frozen aliquots for use in MIC titrations. The addition of 0.1 ml of such dilutions to 2.0 ml of 7H12 broth gave final concentrations ranging from 50.0 to 0.4 μg/ml.

Radiometric method to detect growth. The BACTEC 460-TB instrument (Johnston Laboratories, Towson, Md.) was used to detect the growth of *M. tuberculosis* in 7H12 broth medium (5), which contains ^{14}C-labeled substrates (fatty acids) as a single source of carbon. Growth leads to the consumption of this substrate with subsequent release of $^{14}CO_2$ into the atmosphere above the medium in the sealed

vial. The BACTEC instrument detects the amount of $^{14}CO_2$ and records it as growth index (GI) on a scale of 0 to 999. In the present study, the GI was recorded daily to produce a picture of the dynamics of growth.

Preliminary susceptibility screening. Nine vials of 7H12 medium were inoculated with each culture. A 0.1-ml portion of 7H9 broth culture was used as an inoculum after being adjusted to the optical density of the no. 1 McFarland Standard. This inoculum produced an initial concentration of 5×10^3 to 2×10^4 CFU/ml, as determined by plating. When the GI reached 20 to 50, 0.1-ml portions of the drug solutions (2,000 μg/ml) were added to eight vials to give a final concentration of 100.0 μg/ml for each drug. The ninth vial was a control without drugs. The daily reading by means of the BACTEC instrument continued until a few days after the maximum GI in the control vial was reached. The inhibition, expressed as a ratio of the GI in vials with drugs and in control vials when the GI in the control had reached the maximum, was calculated with the following equation: inhibition = [1 − (GI in drug vial/GI in control vial)] × 100.

R-MIC titration. Ten vials of 7H12 medium were inoculated with each culture under the same conditions as described above. Eight of these vials were used for testing different concentrations of the test drug. Ceforanide was the only drug selected for this study. The appropriate drug solutions (0.1 ml each) were added to the vials once, on day 1 of cultivation. Two vials were used as controls; one was inoculated in the same way as the eight vials with ceforanide and the second was inoculated with a 1:100 dilution of the inoculum to represent 1% of the bacterial population when compared with the other vials. The lowest concentration of ceforanide that completely inhibited the increase in GI, while a daily increase in GI occurred in both controls, was considered the radiometric MIC (R-MIC).

MIC determination in 7H12 broth by plating. Six experiments with different cultures were performed to compare radiometric readings (GI) with the results of plating from the same 7H12 broth cultures (CFU). For this purpose, only two concentrations of ceforanide were selected: one considered the R-MIC in a preliminary experiment with the same culture and the other a concentration which had produced a partial inhibition. Each concentration and control was inoculated in duplicate. Samples for plating were taken at different GI levels in the course of cultivation. An allergist syringe with a fixed, 27-gauge, 0.5-in (1.27-cm) needle (Becton Dickinson and Co., Paramus, N.J.) was used to draw up 0.7 to 0.8 ml of the culture; the plunger was then given a rapid push so that the medium was forced back into the vial. Repetition of this procedure two more times was sufficient to break up most of the clumps. After that, 0.1 ml of the culture was taken as a sample for plating. Each time, the alternate vial in a pair was used for sampling. Two to three dilutions were used for plating (in agreement with preliminary studies) to have a range of 50 to 500 CFU per plate. Four to six plates were used for each sample; each plate was inoculated with a volume of 0.5 ml. The 7H11 agar plates were incubated at 37°C in the presence of 5% CO_2 for 3 weeks; the colonies were then counted.

Susceptibility of ceforanide on 7H11 agar plates. Six concentrations of ceforanide were incorporated in 7H11 agar medium. Two inocula of each culture were used so that there was sufficient growth on the control medium. The MIC in these experiments was defined as the lowest concentration that could produce 99 to 100% inhibition when compared with colony counts on the control medium without ceforanide.

TABLE 1. Results of preliminary screening for susceptibility to 100 μg of eight cephalosporins per ml (33 cultures)

Drug	Distribution of cultures by the level of growth inhibition (%)				
	0–24	25–49	50–79	80–98	99–100
Moxalactam	30	3	0	0	0
Cefoxitin	15	15	3	0	0
Cefamandole	22	6	3	2	0
Cephalothin	15	7	7	2	2
Cefotaxime	0	3	6	10	14
Cephapirin	0	0	0	9	24
Ceftizoxime	0	0	0	4	29
Ceforanide	0	0	1	0	32

Degradation assay. To determine the dynamics of degradation of ceforanide in 7H12 broth cultures, sample vials were removed from incubation at different times: 0, 24, 72, 120, and 192 h. The cultures were filtered through 0.22-μm Millipore filters (Millipore Corp., Bedford, Mass.). The vials without *M. tuberculosis*, containing the same initial concentrations of ceforanide, were also exposed to 37°C and were tested simultaneously with the cultures. The concentration of ceforanide in the filtrate was determined by bioassay, in which a filter paper disk-agar diffusion method with *Bacillus subtilis* ATCC 6633 as a target organism was used (4, 8).

RESULTS

Preliminary screening. Four of the tested drugs (moxalactam, cefoxitin, cefamandole, and cephalothin) did not show significant inhibition of growth in most of the 33 clinical isolates (Table 1). Cephalothin completely inhibited only two cultures (6%). Cephalothin and cefamandole each produced a significant inhibition of two other cultures. In contrast, four other cephalosporins produced significant inhibition of most of the tested cultures. Ceforanide was found to be the most effective, followed by ceftizoxime, cephapirin, and cefotaxime. All cultures included in the column with 99 to 100% inhibition (Table 1) showed not only inhibition but also a significant decrease in GI readings after addition of any of these drugs.

Susceptibility to ceforanide. A total of 65 clinical isolates of *M. tuberculosis* were used for susceptibility studies by two methods: (i) the radiometric method, based on GI readings in the BACTEC system; and (ii) the conventional proportion method, based on comparison of CFU on 7H11 agar plates with and without the drug.

Dose-response relationship in the radiometric method is presented in Fig. 1, in which the percentage of GI inhibition is plotted against ceforanide concentration. The cultures with MIC = 50.0 μg/ml produced the most gentle slope and the cultures with MIC = 6.2 μg/ml gave the steepest slope. The distribution of cultures by the R-MIC in the radiometric method and by the MIC in the proportion method have shown good correlation between these two methods (Table 2). The R-MICs and MICs for most of the isolates were in a range between 6.2 and 25 μg/ml.

No significant differences in susceptibility to ceforanide was found between the cultures susceptible and resistant to the standard tuberculosis drugs. The latter group of isolates consisted of 33 cultures resistant to five to eight drugs and four cultures resistant to only three drugs (isoniazid, rifampin, ethambutol).

Ceforanide degradation in 7H12 broth cultures. The half-life of ceforanide in bacteria-free 7H12 broth at 37°C in the presence of 7% CO_2 in the air above the medium was 75 to

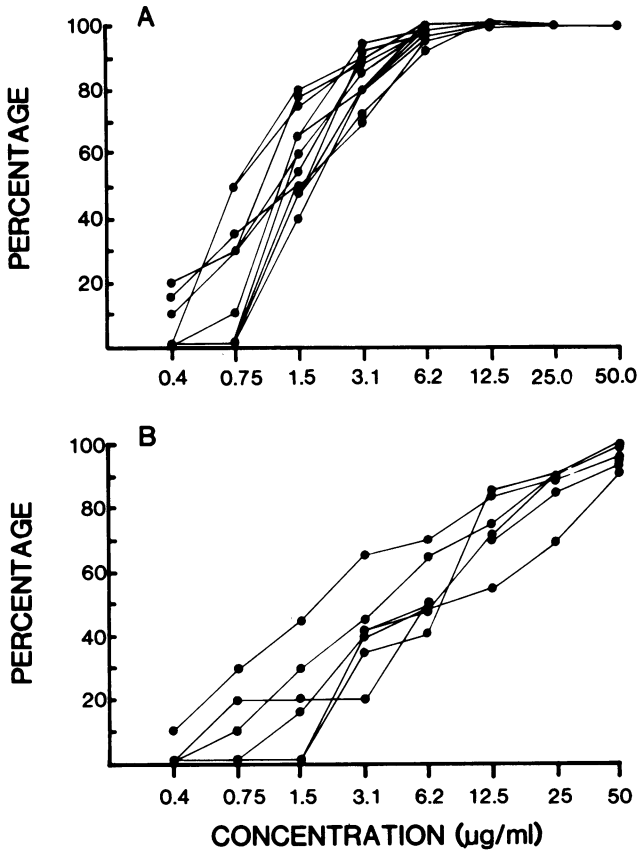

FIG. 1. Dose-response relationships: percentages of inhibition in GIs are plotted against ceforanide concentrations. (A) Cultures with MIC = 6.2 μg/ml; (B) cultures with MIC = 50.0 μg/ml.

83 h (Table 3). To study the degradation in the presence of *M. tuberculosis*, four cultures with R-MIC = 50 μg/ml were selected. The half-life in those conditions depended on the initial concentration of ceforanide. The half-life was the same as in experiments conducted in the absence of *M. tuberculosis*, in which the initial concentration (50 μg/ml) was sufficient for complete growth inhibition. When the initial concentration of ceforanide was lower (25 and 12.5 μg/ml) and the growth of *M. tuberculosis* was inhibited only partially (15 and 50%, respectively), the half-life of ceforanide was shorter (Table 3).

Correlation between GI and CFU dynamics. The typical dynamics of GI, when *M. tuberculosis* is cultivated in 7H12 medium, are shown in Fig. 2A. There was an increase up to the maximum, followed by a decrease. Good correlation between GI and CFU dynamics occurred within the first 10 days of cultivation, until the GI reached the maximum. Within the following period of cultivation, when the GI was declining, the number of CFU continued to rise until it stabilized at 1×10^6 to 2×10^6, which is perhaps the maximum under these conditions. Such a relationship suggests that the observed limit of growth probably was due to the limited amount of nutrient substances in this medium. The decline of the GI while the number of CFU was still increasing could be explained as the result of early consumption of the radiolabeled carbon source occurring before the culture conditions became unfavorable for further multiplication of bacteria. The same course of events took place in the presence of low concentrations of ceforanide, which did not inhibit the growth (Fig. 2B). In the presence of the

TABLE 2. Susceptibility of 65 *M. tuberculosis* isolates to ceforanide, determined by two methods

Ceforanide concn (μg/ml)	Radiometric MIC		Conventional MIC (7H11 plates)	
	No. of cultures	%	No. of cultures	%
0.4	0	—	0	—
0.8	0	—	0	—
1.5	0	—	0	—
3.1	3	4.6	5	7.7
6.2	15	23.0	19	29.2
12.5	26	40.0	16	24.6
25.0	12	18.5	13	20.0
50.0	8	12.3	12	18.5
100.0	0	—	0	—
100.0	1	1.5	0	—

concentration of ceforanide which was considered the MIC, inhibition of growth was detected by both GI readings and CFU counts (Fig. 2C). The same effects were found with all other cultures included in experiments to compare GI and CFU dynamics.

The other type of experiment was conducted to ascertain whether ceforanide produces a bactericidal effect when used in 7H12 broth cultures. From an initial concentration of ca. 10^3 CFU/ml, the cultures were maintained until the increase in GI readings indicated that the culture was in the exponential phase. When the GI reached the level of 100 to 300, ceforanide was added at the concentration considered to be the MIC. In the example shown in Fig. 3, ceforanide was added on day 7 of cultivation, when the GI was 200 and the number of CFU was 2×10^4 to 3×10^4. A significant decrease in both GI and CFU was discovered in the subsequent period of cultivation (Fig. 3B), whereas the events in the control vials (Fig. 3A) reflected the usual dynamics of GI and CFU described above. The decrease in GI after addition of ceforanide remained irreversible, reaching the negative level (<10). At the same time, the decline in CFU occurred within ca. 1 week after the addition of ceforanide, followed by a slight increase within the next period. The number of CFU on day 11 of cultivation was reduced by more than $\log_{10}$ compared with the number of CFU in the same vial at the moment when ceforanide was added (day 7). These data show that ceforanide had a bactericidal effect. The increase

TABLE 3. In vitro degradation of ceforanide in 7H12 broth in the presence and absence of *M. tuberculosis*

Time (h)	Concn of ceforanide (μg/ml):					
	With *M. tuberculosis*			Without *M. tuberculosis*		
Initial (0)	50.0	25.0	12.5	50.0	25.0	12.5
24	38.0	17.5	9.0	38.0	17.5	9.0
72	24.5	11.0	3.5	24.5	14.0	7.0
120	15.0	5.7	2.2	15.0	7.7	3.8
192	11.0	2.8	0.0	11.0	5.8	2.3
Slope[a]	0.0092	0.119	0.0163	0.0092	0.0084	0.0090
Avg half-life (h)	75.3	58.2	42.5	75.3	82.5	77.0

[a] First-order rate constant of degradation: nonlinear regression estimate (h^{-1}). Data best-fitted by one-compartment first-order process. $C_i = A_e^{-\alpha t_i}$, where C_i = concentration at time $t = i$, A = concentration at time zero, α = rate constant of first-order process (equivalent to slope of log concentration-time curve), t = time.

in the number of CFU after a short period of decline was probably due to deterioration of ceforanide (see above) and was a result of multiplication of the part of the bacterial population that was inhibited but not killed after the single addition of ceforanide.

DISCUSSION

Antibacterial activity of ceforanide against *M. tuberculosis* clinical isolates was confirmed by three methods: (i) the dynamics of radiometric readings (GI) in 7H12 broth; (ii) the number of CFU in the same medium; and (iii) the proportion method on 7H11 agar plates. Good correlation among these methods suggests that the radiometric method (BACTEC system) is a reliable tool for such studies. Certain limitations should be taken into account. In the vials without drugs, the dynamics of the GI reflect the dynamics of CFU in 7H12 broth only within the period before the GI readings have reached the maximum. This occurs also in vials with drugs if there is a complete inhibition of GI, which in this case reflects inhibition of growth. At the same time, the decline in GI readings after the maximum was reached in control vials without antibacterial agents does not reflect a decline in the number of CFU (which can continue at the attained level or even increase), but more likely is a reflection of depletion of the radiolabeled nutrient substrate. The decrease in the GI as a result of addition of the antibacterial agent to the culture is a reflection of complete inhibition of growth but does not necessarily reflect the decline in the number of CFU. Therefore the radiometric method may be considered a reliable tool for determining the MIC but not the MBC. With full appreciation of these limitations, the radiometric method is nevertheless a rapid, reliable, and convenient method for preliminary screening of many antibacterial agents and for subsequent MIC titrations of those selected in screening. This method is not labor intensive, is relatively inexpensive, gives immediate results, requires a short cultivation period, and therefore can be used not only in research but also for studies seeking a nonstandard antituberculosis drug in treatment.

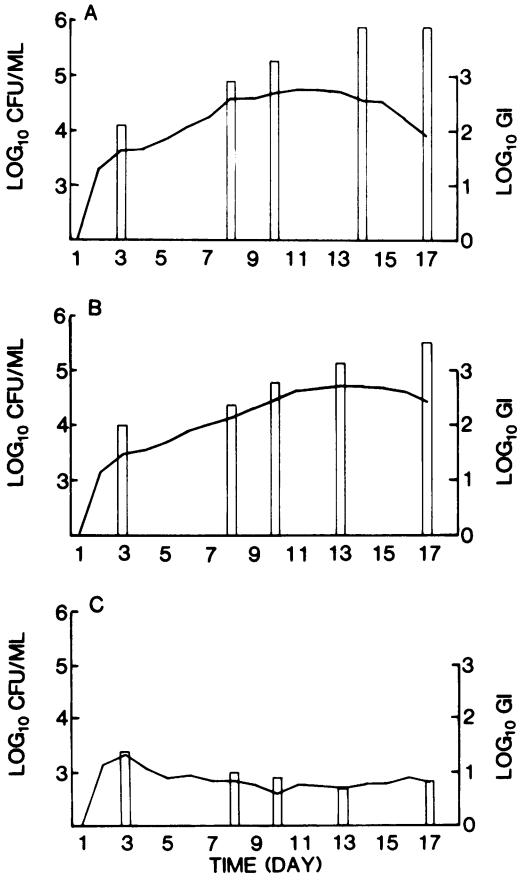

FIG 2. Example of correlation between GIs and CFU dynamics in an experiment in which ceforanide was added to the culture at the beginning of cultivation. (A) Control (without drug); (B) with ceforanide in a low concentration (3.2 μg/ml); (C) with ceforanide in a concentration equal to the MIC (25.0 μg/ml). Bars, CFU; curve, GI daily readings.

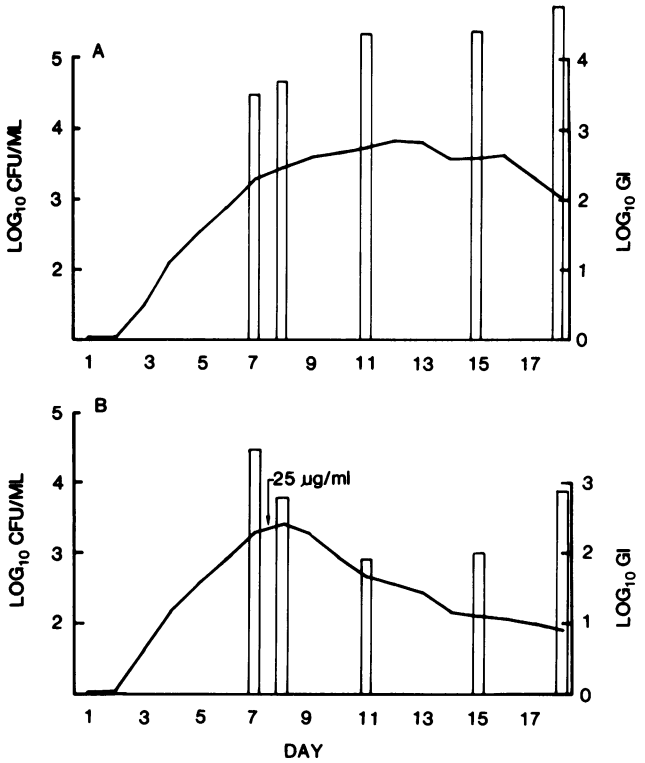

FIG. 3. Example of correlation between GI and CFU dynamics in an experiment in which ceforanide was added at the exponential phase of the growing culture. (A) Control without drug; (B) in the presence of ceforanide in a concentration considered the MIC (25.0 μg/ml). Bars, CFU; curve, GI daily readings.

In this study, the activity of ceforanide against 65 clinical isolates of *M. tuberculosis* corresponds to that found in a previous study in which ceforanide was tested against 12 strains by the broth dilution method (9). The authors found that the MICs were equal to the MBCs, an indication of bactericidal effect. We have confirmed in this study that ceforanide has a clear bactericidal effect when a concentration determined to be the MIC is used in 7H12 broth.

Most of the tested cultures had an MIC in a range between 6.2 and 25.0 μg/ml. Based on the criteria proposed by the National Committee for Clinical Laboratory Standards (1983 tentative standard), more than 60% of the *M. tuberculosis* strains tested in this study by radiometric or agar plate method would be considered susceptible (MIC $\leq$ 8.0) or moderately susceptible (MIC $\leq$ 16.0). However, since these criteria were not derived with mycobacteria, the significance

of these breakpoints for mycobacterial infections remains to be determined.

Whether the observed efficacy of ceforanide in these in vitro assays can be translated into in vivo therapeutic benefit for infections with *M. tuberculosis* remains to be determined. Obviously, such drugs should never be used in routine cases of *M. tuberculosis* infection. However, owing to the increasing incidence of infections with highly drug-resistant organisms (in some cases, resistant to all known standard antituberculosis medications), new approaches to antimicrobial chemotherapy will be required. Development of screening procedures such as those described in this report will facilitate the search for new, potentially useful antimicrobial agents for further testing in animal models and, eventually, in human clinical studies.

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