Determination of Ansamycin MICs for *Mycobacterium avium* Complex in Liquid Medium by Radiometric and Conventional Methods

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A radiometric method to determine the MIC of ansamycin (LM427) for *Mycobacterium avium* complex clinical isolates has been developed. It is based on a comparison of the conventional growth curve determination and the radiometric detection of growth (growth index) in the same liquid medium (7H12 broth). This new method requires less time and labor than does a conventional determination of MIC in liquid medium (CFU). Other advantages of this method include relatively short periods of exposure of the drug to 37°C and the composition of 7H12 broth, which has practically no substrates which could absorb or bind the drug. Thus, a more accurate estimation of the MIC in this medium can be expected than by the conventional agar dilution (proportion) method. The MICs of ansamycin appeared to be higher in agar plates than in 7H12 broth. More than 70% of the isolates had a broth-determined MIC one to three times lower than the average peak concentration of ansamycin achieved in sera of patients. The wide range of MICs suggests the importance of testing susceptibility in broth with many concentrations in addition to, or rather than in, agar plates with concentrations of 2.0 or 1.0 μg/ml only. Taking into account relatively low levels of ansamycin in sera of patients, it would be appropriate to compare the MICs with the levels in serum to make the outcome of chemotherapy more predictable.

Ansamycin (LM427; spiropiperidyl rifamycin) has been reported as having substantial in vitro activity against mycobacteria, especially against *Mycobacterium avium* complex (12, 17). This antimycobacterial agent is widely used in the United States as an experimental drug, primarily for the treatment of disseminated *M. avium* infection in acquired immune deficiency syndrome patients. However, the clinical effect of this treatment is so far unknown. This use of ansamycin is based on in vitro studies which indicated that a high percentage of *M. avium* complex strains were inhibited by 1.0 and 2.0 μg of ansamycin per ml incorporated into 7H10 or 7H11 agar medium. However, several major questions remain to be answered about the utility of this compound in such infections. It is not known whether there is any correlation between the MIC of this drug for the infecting organism in vitro and the clinical (in vivo) response. It is not known whether the concentrations of ansamycin achieved in blood and tissues of patients is sufficient to be effective against strains that are inhibited by different MICs. Is success or failure of chemotherapy a consequence of the relationship between these values? There is a clear need for the development of criteria of susceptibility for in vitro tests which would have a reasonable clinical application. Major questions in this regard are the range of concentrations of ansamycin for in vitro testing, the method to be employed (solid or broth medium), and the development and use of MIC criteria as breakpoints for interpretation. This report is an attempt to approach some of the unsolved problems. Elsewhere (L. B. Heifets and M. D. Iseman, Am. Rev. Respir. Dis., in press) we have reported the results of testing large numbers of *M. tuberculosis* and *M. avium* strains against ansamycin by the agar dilution (conventional proportion) method. The aims of the present study were to determine the range of MICs of ansamycin for *M. avium* clinical isolates in liquid medium, to establish the validity of determining the MIC in 7H12 broth with the radiometric growth index (GI) by comparing such data with the growth curve in the same cultures, to compare results obtained in liquid medium and by the agar dilution method, and to compare the range of MICs with ascertained levels of ansamycin in sera of patients.

We have very limited information about the clinical effects of ansamycin, and only a few of the patients from whom isolates were taken for this study have been treated with ansamycin. Therefore, this report does not present any data regarding the correlation between the results of in vitro testing and the clinical response. The susceptibility studies in this presentation are relevant only to the in vitro inhibition of the bacterial population by different concentrations of the drug.

MATERIALS AND METHODS

Antimicrobial agent. Ansamycin was provided for our studies by Farmitalia Carlo Erba Research Laboratories, Milan, Italy. Stock solutions of 1,000 μg/ml in methanol were kept at −40°C for not more than 2 months.

Cultures. Clinical isolates of *M. avium* complex from National Jewish Hospital patients, as well as referral cultures submitted to National Jewish Hospital for identification and drug susceptibility testing, were the subject of this study. From a total of 211 strains included in this analysis, 97 strains were isolated from patients with acquired immune deficiency syndrome with disseminated *M. avium* infection, and 114 were from patients with localized pulmonary disease. The strains were preserved in 7H9 broth aliquots at −70°C. Results of only one culture per patient were included in this study, and preference was given to the first isolate obtained before treatment. This study analyzes the results

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with subcultures from transparent-type colonies only. For drug susceptibility studies, the strains were subcultivated in 7H9 broth for 2 to 4 days.

MIC determinations in 7H12 broth by the radiometric method. The 7H12 broth medium (10), which contains ^{14}C-labeled substrate (palmitic acid) as a single source of carbon, was used for these studies. Growth leads to the consumption of the substrate, with subsequent release of $^{14}CO_2$ into the atmosphere above the medium in the sealed vial, and the BACTEC TB-460 instrument (Johnston Laboratories, Towson, Md.) detects the amount of $^{14}CO_2$ and records it as GI on a scale of 0 to 999. The GI was recorded daily to produce a picture of the GI curve. An initial vial of 7H12 broth was inoculated with a 1:50 dilution of a 2- to 4-day-old 7H9 broth culture which had been adjusted to the optical density of McFarland standard no. 1. When the GI in this vial reached the maximum (GI 999), this culture was diluted 1:100 and was used to inoculate a set of vials, with 0.1 ml per vial. Our preliminary studies have shown that such an inoculum provides an initial concentration of bacteria in a range between 10^4 and 10^5 CFU/ml. Appropriate working solutions of ansamycin were made from the stock methanol solution in diluting fluid, which contained 0.2% bovine albumin fraction V (Sigma Chemical Co., St. Louis, Mo.) and 0.02% Tween 80. A 0.1-ml volume of a solution added to a 7H12 vial produced an appropriate final concentration of ansamycin. Two vials were used as controls; one vial was inoculated in the same way as the drug-containing vials, and the other vial was inoculated with an inoculum representing 1% of the bacterial population of the other vials (10^2 to 10^3 CFU/ml). Three concentrations (0.25, 0.125, and 0.063 µg/ml) were used for primary titrations, followed by another experiment with an additional set of concentrations if the results in the first experiment were "out of scale." The lowest concentration of ansamycin that inhibited the increase in GI for at least 4 to 5 days while a daily increase in GI occurred in the 1:100 control or which produced a daily GI increase no greater than that in the 1:100 control was considered the radiometric MIC.

MIC determinations in 7H12 broth by plating. The experiments for MIC determination in 7H12 broth by plating were conducted as described above for radiometric MIC determinations, but duplicates and triplicates were used for each concentration, as well as for both controls. Samples from alternate vials were removed for daily plating. Two or three 10-fold dilutions of each sample were used for plating (in agreement with preliminary studies) so as to have a range of 50 to 500 CFU per plate. Four to six 7H11 agar plates were used for each sample; each dilution was inoculated at a volume of 0.5 ml, which was distributed by tilting the plate (not by use of a spreader). Incubation at 37°C was carried out for 12 to 14 days, and the colonies were then counted. The MIC was the lowest concentration that inhibited more than 99% of the bacterial population.

Determinations of the concentrations of ansamycin in broth cultures and sera of patients. To estimate the concentrations of ansamycin in broth cultures, 42 vials of 7H12 broth in each experiment were supplemented with the same concentration of the drug, and half of these vials were inoculated with a strain of *M. avium* resistant to the given concentration. The vials were incubated at 37°C, and duplicate vials both with and without the organisms were removed from the incubator every day during a period of 10 to 12 days. The contents of both vials were filtered through membrane filters (0.22-µm pore size; Millipore Corp., Bedford, Mass.). These samples were stored at −70°C until days 10 to 12, when all

were tested for ansamycin concentration. Levels in serum were determined in patients considered for treatment with ansamycin. Informed consent was obtained from all patients. Antimicrobial agents were withheld for 24 h, and 1 dose of ansamycin (300 mg) was given orally. Blood was collected before the drug was given and then at 2, 4, 6, 12, and 24 h after the loading dose. Each serum specimen was tested twice.

The concentrations of ansamycin in broth cultures and sera were determined by conventional agar plate diffusion bioassay (5, 8) with *Micrococcus luteus* ATCC 9341 as the target organism (8). The standards for experiments with broth cultures were prepared in diluting fluid (see above); for experiments with sera of patients, the standards were prepared in pooled human sera. Results were determined by regression analysis.

Agar dilution method. The study of susceptibility to ansamycin by the agar dilution method was a part of the indirect drug susceptibility test, performed routinely by the proportion method (2, 16) with 12 antituberculosis drugs. Different concentrations of ansamycin (2.0 to 0.015 µg/ml), as well as different concentrations of other drugs, were incorporated into 7H11 agar. Two sets of quadrant petri dishes (one quadrant in each plate contained drug-free medium, and three other quadrants contained the same medium with different drug concentrations) were inoculated with each culture, one with the dilution of 10^{-3} and the other with the dilution of 10^{-5}, made from a broth culture adjusted to the optical density of McFarland standard no. 1. The higher inoculum consisted of 1×10^4 to 3×10^4 CFU; the lower inoculum contained 100 to 300 CFU. Each quadrant was inoculated with 0.1 ml of the bacterial suspension with a repetitive microliter pipette (Eppendorf Repeater 4780; Brinkmann Instruments, Inc., Westburg, N.Y.), and the inoculum was distributed by tilting the plate. Incubation at 37°C in the presence of 5% CO_2 was carried out for 12 days. For most of the isolates, the number of colonies in the controls was in the range of 100 to 300 when the suspension diluted to 10^{-5} was the inoculum. In accordance with the principles of the proportion method (2, 16), the organisms were considered "susceptible" to certain concentrations if the number of colonies in the drug-containing quadrant was less than 1% of the number of colonies in the drug-free quadrant. The lowest concentration that inhibited more than 99% of the bacterial population was considered the MIC determined by this method. The term "susceptibility" is used here to describe only the effects of certain concentrations of the drug on the bacterial population because of paucity of any information about the clinical effect of ansamycin.

RESULTS

MIC determinations in 7H12 broth by radiometric and conventional methods. The initial concentration of bacteria in 7H12 broth (see Materials and Methods) was between 10^4 and 10^5 CFU/ml. Within 4 to 8 days, the number of CFU per milliliter in drug-free medium (undiluted control) reached the maximum of about 10^8 CFU/ml. After a period of stabilization (2 to 4 days), the number of CFU per milliliter began to decline. The maximum CFU of *M. avium* complex strains found per milliliter in 7H12 broth was about 100 times higher than that found with *M. tuberculosis* cultures under the same conditions (9). The increase in daily GI readings correlated well with the growth curve during the period of active growth (Fig. 1A). The subsequent decline in daily GI readings was much sharper and was observed before a reduction
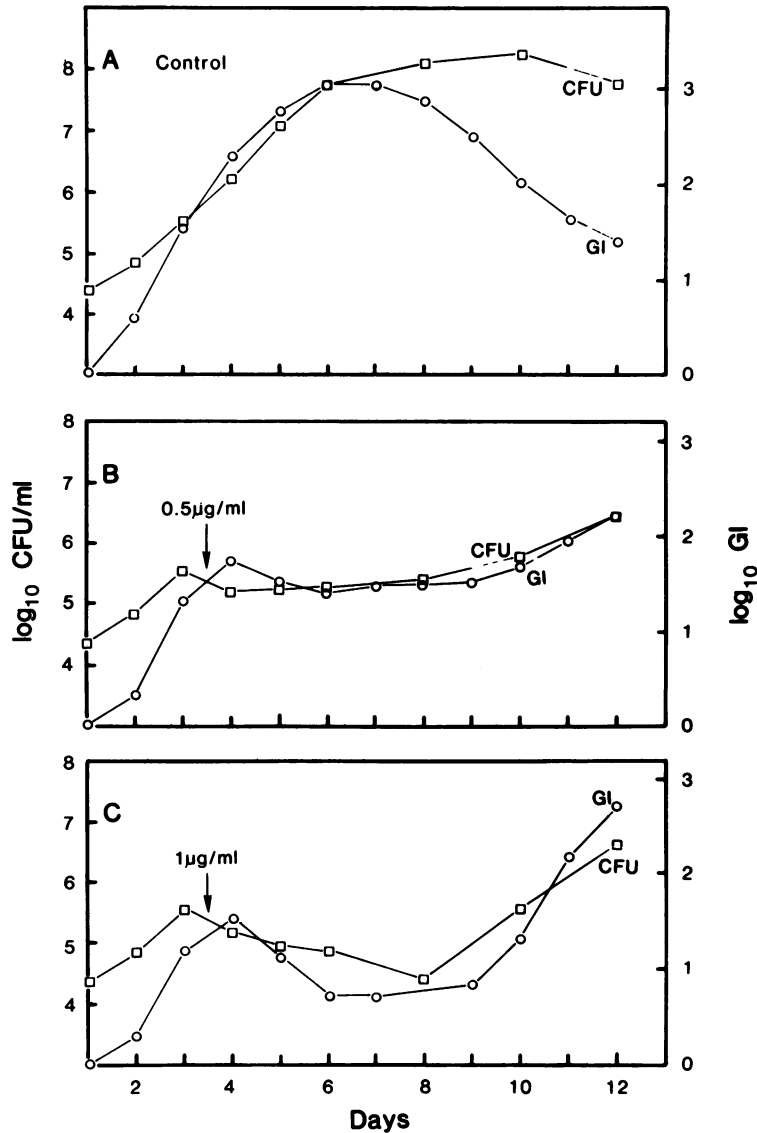
FIG. 1. Example of correlation between the growth curve (CFU per milliliter) and the daily GI readings in an experiment in which ansamycin was added after 3 days of cultivation. (A) Control; (B) with ansamycin at a low concentration (0.5 μg/ml); (C) with ansamycin at a concentration equal to the MIC (1.0 μg/ml) for this strain.

in the number of CFU per milliliter was seen. This course of events had been observed previously for *M. tuberculosis* cultures (9), and it was suggested that the limit of growth in 7H12 broth probably was due to the limited amount of nutrient substances in this medium, consumption of which affects the metabolism of bacteria and the release of $^{14}CO_2$ (GI readings) before the decline in the number of viable organisms can be detected. The growth curves and daily GI reading curves also correlated well in the presence of ansamycin. The subinhibitory concentrations (as in Fig. 1B) delayed the beginning and lowered the intensity of growth detected by both CFU determinations and GI readings. If the lowest concentration considered the MIC inhibited the growth for at least 4 to 5 days, this inhibition was detected by both methods (counting of CFU and GI) and showed good correlation of the curves (Fig. 1C). Very often, such a concentration provided complete inhibition for the first 4 to 5 days only, after which growth was detected by both CFU determinations and GI readings. This phenomenon took

place in experiments in which the drug was added either at the beginning or after a few days of cultivation. These results correspond with the data on deterioration of ansamycin in the medium (see below). The correlation between curves of growth and GI readings in drug-free and drug-containing 7H12 broth was studied with four strains of *M. avium* complex that were inhibited by different MICs of ansamycin. All the experiments confirmed that the daily GI readings truly reflect the multiplication of *M. avium* complex strains in drug-free 7H12 broth during the period of active growth only. The inhibition of daily GI increase in drug-containing vials reflects the true inhibition of growth. These data correspond with our previous finding in regard to the use of a GI criterion for *M. tuberculosis* as well as (9). It is essential in radiometric MIC titration to compare the curve of GI daily readings in drug-containing vials with that of the 1:100 control, as defined above. The daily GI increase in the vial with the concentration considered the MIC was less than that in the 1:100 control, which is an indication that less than

TABLE 1. MICs of ansamycin for 211 *M. avium* isolates as determined by two methods

Ansamycin concn (μg/ml)	Radiometric method (7H12 broth)		Conventional method (7H11 agar plates)	
	No. of cultures	% of strains	No. of cultures	% of strains
0.015	6	2.8	0	0
0.031	5	2.4	4	2.0
0.062	32	15.2	3	1.4
0.125	65	30.8	14	6.6
0.25	56	26.5	37	17.5
0.5	37	17.5	66	31.3
1.0	8	3.8	49	23.2
2.0	2	1.0	38	18.0

1% of the bacterial population was resistant to this concentration, as defined in the so-called radiometric proportion method (11, 13–15).

Comparison of the results obtained by broth and agar dilution methods. A total of 211 strains were tested by both broth and agar dilution methods. The agar dilution test was performed as part of the routine drug susceptibility test with 12 antituberculosis drugs and was then repeated simultaneously with the experiments in broth. The radiometric MIC determination was done twice with about half of the strains. These repeated studies showed high consistency in results for both methods; when MIC titrations were repeated, they

never varied by more than 1 dilution. Most of the isolates appeared more susceptible to ansamycin in 7H12 broth than on 7H11 agar plates; in broth, about 78% of the strains were inhibited by MICs of 0.25 μg/ml or less, whereas less than 28% of the same strains were inhibited by this range when they were tested in 7H11 agar medium (Table 1). The MICs for 50% of the strains were calculated as 0.75 μg/ml for the agar method and 0.35 μg/ml for broth. The MICs for 90% of the strains were calculated 1.53 and 1.25 μg/ml, respectively. The results obtained in this study by the agar dilution method were in good agreement with our previous findings (Heifets and Iseman, in press), with a larger number of strains (523) tested by the same method from 1983 through 1984.

Despite the different levels of susceptibility found by the agar and broth methods, the majority of strains considered among the most susceptible by the agar method were found to be the most susceptible by the broth method, and the most resistant strains by the agar method were also the most resistant strains by the broth method (Fig. 2). Among 58 strains with MICs of 0.25 μg/ml or less determined in agar, 82.8% had broth-determined MICs of 0.125 μg/ml or less. Among 38 strains with MICs of 2.0 μg/ml or more by the agar method, 85.5% had broth-determined MICs of 0.25 μg/ml or more.

Ansamycin degradation in 7H12 broth cultures. The half-life of ansamycin in bacteria-free 7H12 broth at 37°C in the presence of 7% CO_2 in the air above the medium was 157 to 197 h (Table 2). The half-life was the same in the presence of
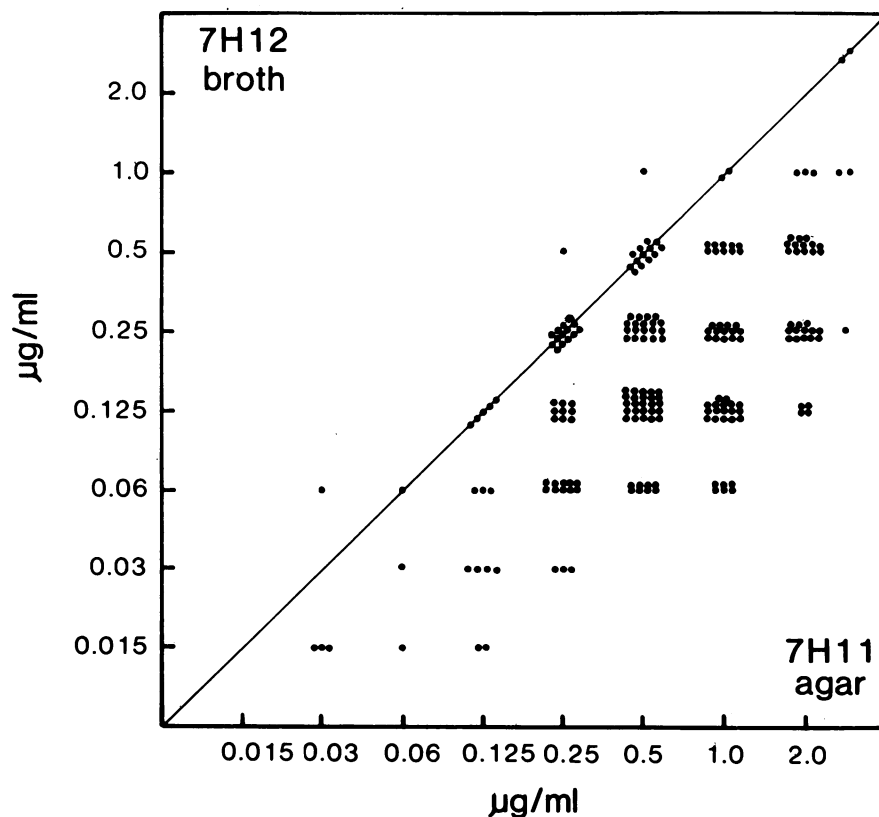

FIG. 2. MICs of ansamycin determined by 7H12 broth and 7H11 agar plate methods.

TABLE 2. In vitro degradation of ansamycin in 7H12 broth at 37°C (average of three experiments)

Initial concn (μg/ml)	Concn (μg/ml) of ansamycin at h:										Regression analysis	
	24	48	72	96	120	144	168	192	216	240	Slope	Half-life (h)
2.0	1.93	1.58	1.69	1.33	1.25	1.07	0.96	0.96	0.95	0.63	0.0044	157
1.0	0.87	0.78	0.78	0.67	0.67	0.50	0.50	0.50	0.46	0.43	0.0035	197

M. avium strains completely or partially resistant to the concentrations of the drug chosen for this study.

Levels of ansamycin in sera of patients. Peak levels of drug in serum were achieved 4 h after the administration of 300 mg of ansamycin (Fig. 3). The average peak level was 0.38 μg/ml (0.25 ÷ 0.58), which approximates information presented by the manufacturer of the drug (0.49 μg/ml).

DISCUSSION

Employment of 7H12 broth offers an opportunity to compare the results of MICs determined by conventional sampling and plating (CFU) with results of daily radiometric detection of growth (GI). Previously (9), such comparisons showed good agreement when the MICs of cephalosporins against *M. tuberculosis* were determined. In the present study, good correlation between the growth curve and GI daily-reading curve was found in the determination of ansamycin MICs for *M. avium* complex strains. These results led to the establishment of a radiometric determination of the MIC, which is a fast, convenient, and reliable method requiring much less time and labor than does a conventional determination in liquid medium. This approach was essential in this study, which was to determine the range of MICs of ansamycin by testing a substantial number of *M. avium* complex strains. MIC determination by the radiometric method presents certain limitations respecting the inoculum size, which must provide an initial concentration of *M. avium* organisms in range of 10^4 to 10^5 CFU/ml. Under these conditions, the growth in drug-free medium (undiluted control) achieves its maximum GI within no less than 4 days and after no more than 8 days, the optimum time span for detection of ansamycin activity. Another requirement is that an additional control with a 1:100 inoculum (10^2 to 10^3 CFU/ml) be used. The lowest concentration which produces a daily GI increase no greater than that in the 1:100 control is the MIC, which, in other words, is the lowest concentra-

tion inhibiting growth of more than 99% of the bacterial population (11, 13–15).

The comparison of the MICs detected in 7H12 broth and by the agar plate method has demonstrated that most of the strains were much more susceptible when the test was done in liquid medium. One of the possible explanations for this phenomenon is the differences in experimental conditions in agar and broth. Difference in the inoculum size, known to be a cause of different results of susceptibility in agar and broth media, was not likely the case in this study. For the agar plate method, two inocula were used to determine if a strain contained 1% drug-resistant mutants; the higher inoculum consisted of 1 × 10^4 to 3 × 10^4 cells (CFU), and the lower inoculum contained 100 to 300 CFU per drug-free or drug-containing plate quadrant. For the broth method also, two inocula were used for the drug-free media (two controls); the higher inoculum produced an initial concentration of 10^4 to 10^5 CFU/ml, and the lower inoculum (1:100 control) produced a concentration of 100 to 1,000 CFU/ml. The drug-containing vials were inoculated with the higher inoculum only. So the lower MICs in broth were obtained despite the fact that the inoculum in broth was not lower than with agar plates. The more likely explanation for the differences in MICs between agar and broth methods is the instability of the drug during the incubation at 37°C. In our previous studies with more stable drugs (9), no significant differences in MICs were found between the agar and broth methods. The results were different in this study with ansamycin. The agar plate method requires at least 12 days of incubation to obtain visible growth in controls and to detect the effect of inhibition, whereas the effect in 7H12 broth can be detected by radiometric readings within a few days of the addition of the drug. The actual active concentrations in the agar medium are probably much lower than those originally incorporated into the agar, due to the deterioration during a longer period of incubation. This assumption is in concordance with the observed results of degradation of ansamycin at 37°C even in liquid medium. Therefore, the determination of ansamycin MICs by the agar dilution method can be accepted only conditionally. Susceptibility testing in 7H12 broth more accurately reflects the MICs, since this method requires only a few days for detection of the inhibitory effect. Alternatively, the drug could be added after 2 or 3 days of incubation, thus shortening the period of exposure of the drug to 37°C.

The average peak level of ansamycin in serum after administration of 300 mg was 0.38 μg/ml; this level corresponds with previous findings (4). A peculiarity of ansamycin is that its concentration in human tissues is reported to be 10 to 20 times higher than in serum (4). The accepted criteria of susceptibility of *M. tuberculosis* to antituberculosis drugs with regard to the concentrations incorporated into solid media were developed without any direct reference to the achievable levels in blood, but rather to the correlations between the clinical response and results of in vitro inhibition produced by certain concentrations of drugs
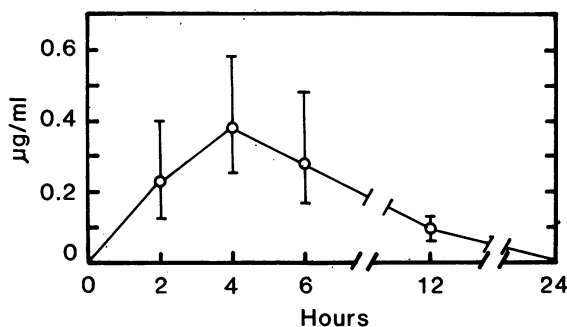

FIG. 3. Levels of ansamycin (micrograms per milliliter) in sera of 7 patients after oral administration of 300 mg of drug (geometric means and standard deviations).

incorporated into different solid media (2). The results of such in vitro testing with *M. tuberculosis* helped predict the clinical response, and many years of experience in different countries proved that this approach was very helpful in monitoring the chemotherapy of tuberculosis. The situation with chemotherapy of *M. avium* infection is quite different. There are no reports about correlation between in vitro drug susceptibility testing results and clinical responses in patients with this disease. Multidrug chemotherapy was successful in 60 to 80% of cases (3, 7) despite the fact that, when results were interpreted by criteria developed for *M. tuberculosis*, most of the *M. avium* clinical isolates appeared to be resistant to most conventional antituberculosis drugs. The development of in vitro susceptibility criteria with different drugs for *M. avium* infection is an obvious necessity. The results of in vitro studies with ansamycin showed that this agent appeared to be much more active against *M. avium* strains than were the conventional antituberculosis drugs. The clinical effect of ansamycin is so far unknown. The question is, what kind of in vitro data should be compared with the clinical response to develop criteria of susceptibility? Unlike the situation in the 1950s and 1960s, when criteria of susceptibility of *M. tuberculosis* were developed, it is possible now to use not only solid media, but a liquid medium as well (7H12 broth) in determining the MICs for clinical isolates. If the levels of susceptibility to ansamycin in agar plates obtained in this study were taken as the true MICs, the data indicate that the susceptibility of most isolates is at 0.5 μg/ml or more, a level which cannot be translated into an attainable concentration in serum in most patients and from which a suboptimal clinical response might be expected in most cases. If the levels of susceptibility in broth were taken as the MICs, then 70% or more of the patients could achieve a level in blood (and especially a level in tissue) equal to one or more MICs. The broad range of MICs for *M. avium* clinical isolates suggests that the responses to chemotherapy with ansamycin would be quite unpredictable. It seems appropriate, therefore, to determine the exact level of susceptibility, rather than to screen the strain at 1.0 and 2.0 μg/ml only. Even by the conventional agar plate method, the concentration of 0.5 μg/ml should be used in addition to concentrations of 1.0 and 2.0 μg/ml. Considering the observed low levels of ansamycin in serum, we believe that it would be useful to compare the clinical response with the MICs determined not only by the agar dilution method, but in liquid medium as well (the radiometric method is a convenient tool for this comparison). This approach would provide a rational basis from which to predict the efficacy of therapy with ansamycin and develop the criteria of clinical susceptibility to this drug for *M. avium* organisms. It will be worthwhile to try to interpret MIC data by using the inhibitory quotient, which is a number reflecting the multiple of the MIC that could be achieved in blood or tissues (6). Obviously, since this methodology is new, its utility must be tested in a clinical study.

ACKNOWLEDGMENTS

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