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Susceptibility of *Mycobacterium malmoense* to Antibacterial Drugs and Drug Combinations

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***Mycobacterium malmoense* is an opportunistic pathogen with increasingly recognized clinical importance. It is mainly isolated in northern Europe and Great Britain, most often from patients with pulmonary infections. Conventional therapy of *M. malmoense* infections with antituberculosis drugs is often of limited value, and there is thus a need for improved drug regimens. The potential efficacies of new alternative drugs, such as quinolones, macrolides, amikacin, and rifabutin, are still unknown, and so is the pathogen's in vitro susceptibility to most of these drugs. In this study, we used the BACTEC system for determining the pattern of resistance of clinical *M. malmoense* isolates to a number of antibacterial drugs as well as their possible synergistic interactions when each of them was combined with ethambutol. The majority of the strains were resistant or moderately resistant to the drug when it was tested alone at selected concentrations. However, pronounced in vitro synergism was demonstrated for combinations of ethambutol with ciprofloxacin, amikacin, and rifampin, rendering most isolates susceptible to the combined drugs. Thus, for in vitro susceptibility testing of *M. malmoense*, examination of the possible synergistic effects of combined drugs also can be recommended.**

Mycobacterium malmoense is a clinically relevant, slowly growing, nonpigmented mycobacterial species. It was first described in 1977 by Schröder and Juhlin (24), who reported four cases of pulmonary infection in southern Sweden. *M. malmoense* is isolated in increasing numbers from clinical samples in northern Europe and Great Britain and is today, next to *Mycobacterium tuberculosis* and *Mycobacterium avium* complex, the most common cause of mycobacterial infection in Sweden (10). Like most other atypical mycobacteria, it has a high degree of resistance to antibacterial drugs, including specific antituberculosis drugs such as isoniazid, *p*-aminosalicylic acid, pyrazinamide, and ethambutol.

Today there is still no generally accepted in vitro assay for testing the drug susceptibility of *M. malmoense* or related mycobacterial species. There are, however, strong reasons to believe that methods based on culture in liquid media give the most relevant results, an idea which has been discussed by Heifets et al. for the testing of *M. avium* complex (9). We have previously shown that the BACTEC system (Becton Dickinson, Cockeysville, Md.) allowed good growth of *M. malmoense* and thereby significantly improved its rate of primary isolation from clinical samples (12). In the present study, we used the BACTEC method for testing in vitro the susceptibilities of 22 strains of *M. malmoense* to the antibacterial agents ethambutol, ciprofloxacin, rifampin, rifabutin, and clarithromycin. Previously we have demonstrated pronounced antibacterial effects of certain combinations of antibacterial drugs against *M. avium* complex (14–16) and *Mycobacterium kansasii* (11). On the basis of these studies, we have suggested that ethambutol has a key role as a potentiator in antimycobacterial drug combinations (18). In this study, we therefore investigated the susceptibility of *M. malmoense* not only to certain antibacterial drugs used alone but also to the combination of each of them with ethambutol.

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MATERIALS AND METHODS

Strains. A reference strain, ATCC 29571, and 21 clinical isolates of *M. malmoense* were included in the study. Of the isolates, 13 were derived from patients with pulmonary infections and 8 were derived from lymph nodes of children with lymphadenitis.

The strains were identified by standard biochemical tests (20) and especially by their capacity to hydrolyze Tween 80, which was used to distinguish them from the related *M. avium* complex.

Drugs. The following drugs used were obtained as powders with stated potencies: ethambutol, batch 435M (Lederle, Wayne, N.J.); rifabutin, batch T01013 (Farmitalia Carlo Erba, Milan, Italy); rifampin, batch 9038086 (Ciba-Geigy Ag, Basel, Switzerland); ciprofloxacin, batch 300459 (Bayer AG, Leverkusen, Germany); amikacin, batch MR 7126 (Bristol-Myers Squibb Co., New York, N.Y.); and clarithromycin, batch 76-343-AL (Abbott Laboratories, North Chicago, Ill.). A stock solution of each drug was prepared in 0.067 M phosphate buffer, pH 7.2, with a concentration 40 times the final test concentration used. Rifabutin and rifampin were dissolved in small amounts of dimethyl sulfoxide before the buffer was added. To each culturing vial containing 4 ml of medium, 0.1 ml of the stock solution was added to give the following test concentrations: ethambutol, 5.0 mg/liter; rifampin, 0.25 mg/liter; rifabutin, 0.25 mg/liter; ciprofloxacin, 2.0 mg/liter; amikacin, 4.0 mg/liter; and clarithromycin, 4.0 mg/liter. These concentrations were established by using data from studies of the inhibitory effects of serial dilutions of each drug (data not shown) and by taking the achievable serum drug concentrations into account.

Susceptibility testing. The BACTEC radiometric system (Becton Dickinson) and 7H12B Middlebrook TB medium, an enriched 7H9 broth supplemented with bovine serum albumin, catalase, casein hydrolysate, and ^{14}C-labeled palmitic acid (22), was used. The tested strains were subcultured on Löwenstein-Jensen egg medium at 37°C for 3 to 4 weeks before being suspended in sterile phosphate-buffered saline (PBS), pH 7.2, to give a bacterial density corresponding to a McFarland standard of 0.5, which after a further 1/10 dilu-

TABLE 1. Susceptibilities of 22 strains of *M. malmoense* to various antibacterial drugs at clinically attainable concentrations

Drug	Concn (mg/liter)	No. of strains		
		Susceptible	Moderately resistant	Resistant
Ethambutol	5.0			22
Amikacin	4.0		9	13
Ciprofloxacin	2.0	1	3	18
Clarithromycin	4.0	19		3
Rifabutin	0.25	9	9	4
Rifampin	0.25		1	21

TABLE 2. Susceptibilities of 22 strains of *M. malmoense* to the combination of ethambutol with other antibacterial drugs

Drug combined with ethambutol[a]	Concn (mg/liter)	No. of strains		
		Susceptible	Moderately resistant	Resistant
Amikacin	4.0	6	16	
Ciprofloxacin	2.0	9	13	
Clarithromycin	4.0	19		3
Rifabutin	0.25	15	6	1
Rifampin	0.25	2	18	2

[a] Ethambutol was used at a concentration of 5 mg/liter.

tion in PBS gives a final concentration in the test vials of approximately 10^5 CFU/ml (16). An aliquot of 0.1 ml of the diluted suspension was inoculated into each culturing vial containing a drug or drug combination, as well as into one drug-free control vial. A 1/100 dilution of this bacterial suspension was used as the inoculum in a second control vial. The $^{14}CO_2$ produced by the metabolically active mycobacteria was quantified daily with a BACTEC 460 instrument (Becton Dickinson) over a 4-day period. The results were expressed as growth index (GI) values ranging from 0 to 999.

The principle for the radiometric evaluation of drug interactions and for determining mycobacterial susceptibility to combined drugs has been reported earlier by us (15, 16). The interpretation of the susceptibility tests is based on a comparison of the growth of a drug-exposed culture registered on day 4 with both the corresponding value registered the preceding day and the growth in unexposed control cultures. An isolate was defined as susceptible when the radiometric GI value on day 4 [GI(4)] of a drug-exposed culture was less than or equal to the GI(3), thus revealing reduced metabolic activity in the culture. When there was an increase in metabolic activity, reflected by increasing GI values from day 3 to day 4, the isolate was defined as resistant to the tested drug. As an intra-assay control, the GI(4) of the test vial was divided by the GI(4) of the diluted control vial. If a strain regarded as susceptible yielded a quotient of >0.5 (reflecting a high level of metabolic activity), it was deemed moderately resistant. Similarly, if a strain regarded as resistant showed a quotient of 0.05 (reflecting a low level of metabolic activity), it also was deemed moderately resistant.

RESULTS

The majority of the *M. malmoense* strains were resistant or moderately resistant to all but one of the drugs when the drugs were tested separately (Table 1). The exception was clarithromycin, which inhibited 19 of 22 strains at a concentration of 4 mg/liter. Of these 19 strains, 6 were also tested against lower drug concentrations; 5 of the 6 were also susceptible to 1 mg of clarithromycin per liter. In a pilot study, the antimycobacterial effect in vitro of clarithromycin was compared with that of another macrolide compound, roxithromycin. Clarithromycin was the more effective of these two, since five of six and six of six *M. malmoense* strains were inhibited by 1 and 8 mg of clarithromycin per liter, respectively, whereas zero of six and two of six of these strains were susceptible to the same concentrations of roxithromycin.

Compared with the generally poor results obtained with single drugs, drastically increased antimycobacterial effects were seen for combinations of ethambutol with each of the other agents (Table 2). This effect was most pronounced for combinations of ethambutol with ciprofloxacin, amikacin, and rifabutin, for which the number of susceptible strains increased by 8, 6, and 6 strains, respectively.

The pattern of resistance of the *M. malmoense* reference strain ATCC 29571 to single and combined drugs did not differ from that of recent clinical isolates. The lymph node isolates were identical to the pulmonary isolates in their responses to the antibacterial drugs tested, with the exception of rifabutin, to which many of the (earlier often drug-exposed) pulmonary isolates tended to be less susceptible. This was true both when rifabutin was used alone and when it was tested in combination with ethambutol.

DISCUSSION

The clinical significance of *M. malmoense* is increasingly recognized. Improved methods for the laboratory identification of this bacterium (12) are reflected by a steady increase in diagnosed cases. Geographically, most cases are found in Great Britain and parts of northern Europe, mainly Sweden (10, 12) and Finland (19). However, increasing numbers of case reports from the United States (1, 26) and several other countries in Europe (2–7, 17, 19, 21, 23, 25) indicate that disease caused by *M. malmoense* is not geographically limited.

Little is known today about the optimal drug treatment of *M. malmoense* infections. There are no controlled clinical trials to study the efficacies of various drug regimens or individual drugs or to evaluate the effectiveness of chemotherapy in general. Information thus has to be drawn from a limited number of case reports, each discussing a limited number of patients retrospectively analyzed. Banks and coworkers reported in 1985 that five patients with pulmonary *M. malmoense* infections responded to an 18- to 24-month-long treatment period with a three-drug combination of rifampin, isoniazid, and ethambutol, while relapses occurred in five other patients on shorter treatment regimens (2). In particular, the discontinuation of ethambutol in this regimen seemed to correlate with relapse. This drug combination has since often been considered a standard regimen for *M. malmoense* infections. We question the usefulness of in vitro susceptibility tests because of an apparent lack of correlation between the results of such tests and the clinical outcome. This could at least in part be explained by the fact that only conventional testing of susceptibilities to single drugs was performed, thereby not revealing the probably significant antibacterial drug interactions.

In the present study, clinical data on response to drug therapy were obtained retrospectively from 7 of the 13 patients with lung infections included in the study. Different

drug regimens were used, but all included ethambutol and rifampin (for 6 months and up to 2 years), a combination demonstrated in vitro to have a strong antimycobacterial effect. All seven patients responded clinically to the treatment, thus supporting a correlation between the in vitro result and the clinical outcome.

Synergistic or additive effects, as defined for inhibitory antibacterial interactions of combined drugs against *M. avium* complex (16), were seen with most strains in this study, not only for the combination of ethambutol with rifampin but also for ethambutol combined with ciprofloxacin or rifampin.

The importance of ethambutol in the regimen for pulmonary *M. malmoense* infections, suggested by these in vitro results, is stressed by France et al. (7). It is further supported by the fact that pronounced clinical improvement was noted when ethambutol was added to the antimycobacterial regimen given to an AIDS patient with concomitant *M. malmoense* infection (4).

M. malmoense strains are comparatively susceptible to rifamycins. In an earlier study, 60% of the *M. malmoense* strains tested were susceptible in vitro to 1.0 mg of rifampin per liter (8). In this study, we used a low concentration (0.25 mg/liter) of each rifamycin. At this concentration, rifabutin was significantly more effective against *M. malmoense*, with 9 of the 22 strains being susceptible, while none of them was inhibited by rifampin (Table 1). We have previously reported the lack of inhibitory effect of isoniazid (alone or combined with ethambutol) on *M. malmoense* in vitro. Increased growth of *M. malmoense* was seen in the presence of isoniazid at a concentration of 0.2 mg/liter (13).

In vitro resistance to the antituberculosis drugs *p*-aminosalicylic acid, pyrazinamide, thiacetazone, and ethambutol is reported also (8). In the present study, all strains tested were resistant to 5 mg of ethambutol per liter. A potentiation by ethambutol of the antibacterial effects in vitro against *M. malmoense* for a number of other antibacterial agents, including aminoglycosides, rifamycins, fluorinated quinolones, and macrolides, was shown in this study. This corresponds to what we and others previously reported for *M. avium* complex (for example, see references 14–16 and 27) and *M. kansasii* (11). Since such synergistic drug interactions against *M. malmoense* occurred with several of the drug combinations tested, it seems clear to us that in vitro susceptibility tests should also include evaluations of possible antimycobacterial effects of combined drugs. In our opinion, this is true also for other atypical mycobacteria.

To what extent these promising antimycobacterial in vitro results correlate with clinical efficacy and thus make possible an improved drug therapy of *M. malmoense* infections merits further evaluation.

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