Journal of Antimicrobial Chemotherapy (1995) **36**, 497–502

Activity of seven antimicrobial agents, alone and in combination, against AIDS-associated isolates of *Mycobacterium avium* complex

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The activity of seven antimicrobial agents (and five two-drug combinations and five three-drug combinations) was investigated against 37 clinical isolates of *Mycobacterium avium* recovered from blood cultures of AIDS patients. The susceptibility tests were performed in Middlebrook 7H12 broth using a radiometric method. MICs of amikacin, ciprofloxacin, clarithromycin, clofazimine, ethambutol, rifabutin and sparfloxacin were determined. Five antimicrobial agents were tested in combination with clarithromycin and also with clarithromycin plus amikacin to look for possible synergic activity. Synergic activity in combination with clarithromycin and with clarithromycin plus amikacin, was detected for rifabutin (54% and 51% of isolates, respectively), clofazimine (38% and 35%), ethambutol (16% and 32%), ciprofloxacin (8% and 14%) and sparfloxacin (3% and 8%). No antagonism was observed. We conclude that clarithromycin is an essential component in the chemotherapy of *M. avium* complex disease.

Introduction

Disseminated *Mycobacterium avium* complex (MAC) infection is common in AIDS patients, occurring in 40–60% of subjects (Nightingale *et al.*, 1992; Benson, 1994*a*). Although infection with this organism arises late in the course of the disease, when other opportunistic infections usually have already been diagnosed, it causes disabling symptoms and results in shortened patient survival (Chin *et al.*, 1994). Chemotherapy of MAC infection is hindered by the intracellular location of the organism and its increased resistance to most antimycobacterial agents (Inderlied, Young & Yamada, 1987; Rastogi, 1993). In this respect, it is now well established that antibiotic resistance in MAC is closely associated with the cell envelope, which acts as a barrier inhibiting drug penetration (Rastogi *et al.*, 1981). In an attempt to circumvent this problem, many new antimicrobial agents and their combinations are being evaluated for in-vitro activity (Grosset, 1994).

At present it seems likely that better control of MAC disease will need multiple-drug therapy. The purpose of the present multicentre study was to investigate the in-vitro

0305–7453/95/090497 + 06 $12.00/0 © 1995 The British Society for Antimicrobial Chemotherapy

susceptibility of MAC strains isolated in Italy and to determine the most effective antibiotic combinations.

Methods

Study sites

The study was done at five centres: the "Umberto I°-Torrette" Hospital, Ancona; the Careggi Hospital, Florence; the Institute of Infectious Diseases, "La Sapienza" University, Rome; the United Hospitals, Bergamo; and the Institute of Microbiology, Perugia University, Perugia.

Test strains

Thirty-seven *M. avium* strains isolated from the blood of AIDS patients and identified to species level by RNA-DNA hybridization (Gen-Probe, San Diego, USA), were studied. Smooth, transparent colonies growing on Middlebrook 7H10 agar were sub-cultured for MIC and synergism determinations.

Antimicrobial agents

Clarithromycin (Abbott Laboratories, North Chicago, USA), clofazimine (Ciba-Geigy, Basel, Switzerland), rifabutin (Farmitalia-Carlo Erba, Milan, Italy), ciprofloxacin (Bayer Pharmaceuticals, Leverkusen, Germany) and sparfloxacin (Rhône Poulenc-D.P.C. Europe, Antony, France) were kindly provided by the manufacturers. Amikacin, and ethambutol were purchased from Sigma Chemical Co. (St Louis, USA).

A stock solution of clarithromycin was made in methanol and then diluted with phosphate buffer at pH 6·8 made by combining 0·1 M solution of KH_2PO_4 and Na_2HPO_4. Stock solutions of clofazimine, rifabutin and sparfloxacin were made, respectively, in dimethyl sulphoxide (DMSO), methanol and 1 N NaOH. Stock solutions of amikacin, ethambutol and ciprofloxacin were made in distilled water and then sterilized using a membrane filter (pore size 0·22 μm; Millipore Corp., Bedford, USA). All stock solutions were kept in aliquots at -70°C, except for clofazimine which was stored at room temperature in the dark. Working solutions, the concentrations of which were 40-fold greater than the desired concentrations, were made from stock solutions in sterile distilled water, except for clofazimine which was diluted in DMSO.

Radiometric method

The growth of bacteria was recorded radiomerically using the BACTEC 460-TB system (Becton Dickinson, Sparks, USA). Growth in Middlebrook 7H12 liquid medium (Becton Dickinson) containing ^{14}C-labelled palmitic acid leads to the consumption of this substrate, with subsequent release of $^{14}CO_2$ in the confined atmosphere above the medium (Middlebrook, Reggiardo & Tiggert, 1977). The BACTEC instrument detects the amount of $^{14}CO_2$ and records it as a Growth Index (GI) on a scale from 0 to 999.

MIC determination

For broth-determined MICs, all the antibiotics were added in 0·1 mL volumes to respective Middlebrook 7H12 vials to achieve serial doubling dilutions. A Middlebrook 7H12 broth seed vial was incubated and monitored daily until it reached the maximum GI, and the culture was then diluted 1:100 and 0·1 mL were inoculated in the test vials and in a drug-free control vial. This represented an initial concentration of 10^4–10^5 cfu/mL. Another drug-free control vial, the 1:100 control, was inoculated to achieve an initial concentration of 10^2–10^3 cfu/mL. The vials were incubated at 37°C and the GIs were recorded daily. According to previous studies (Heifets, 1991*a*; Siddiqui *et al.*, 1993), the MIC determined by the BACTEC system is defined as the lowest antibiotic concentration in the presence of which the final GI reading was $\leqslant 50$ after incubation for 8 days. During the same period the GI of the 1:100 control was >20 on three consecutive days, while the growth in the undiluted control reached the maximum GI (999) no earlier than the fourth day of incubation.

MIC validation

To verify that the MIC was the lowest concentration inhibiting the growth of more than 99% of the bacterial population, at one of the test centres we serially diluted aliquots of broth, plating on to Middlebrook 7H10 agar.

Combined antibiotic activity

Ciprofloxacin, clofazimine, ethambutol, rifabutin and sparfloxacin were tested in combination with clarithromycin (two-drug combinations) and with clarithromycin plus amikacin (three-drug combinations). Combined antibiotic activity was evaluated by calculating the fractional inhibitory concentration (FIC) index (Heifets, 1991*b*). Doubling dilutions of each antibiotic were added to the Middlebrook 7H12 broth vials to represent 1/2, 1/4, 1/8, and 1/16 MIC of all drug combinations. The technique was the same as that described for MICs determination. For the two-drug combinations, a FIC index $\leqslant 0{\cdot}5$ and >2 was considered to represent synergy and antagonism, respectively. A FIC index $\leqslant 0{\cdot}75$ indicated synergy when testing three-drug combinations.

Results

The activities of seven antimicrobial agents are summarized in Table I. Except for amikacin and clofazimine, the MICs for most of the strains were high in comparison with the concentrations achievable in serum with standard dosages.

FIC indices for each of the two-drug combinations against 37 MAC strains are shown in Table II. Synergy was observed when clarithromycin was combined with rifabutin (54% of strains), clofazimine (38%), and ethambutol (16%). The combinations of clarithromycin and rifabutin appeared to be the most effective (11% of MAC strains with FIC index $\leqslant 0{\cdot}25$). The other combinations basically showed additive effects; no antagonism was recorded.

FIC indices for each of the three-drug combinations against 37 MAC strains are shown in Table III. Synergism was observed when clarithromycin plus amikacin were combined with rifabutin (51%), clofazimine (35%) and ethambutol (32%). However, while the

Table I. Activity of seven antimicrobial agents against 37 *M. avium* strains

	MIC_{50} mg/L	MIC_{90} mg/L	Serum concentrations[a]
Amikacin	4	8	20–30
Ciprofloxacin	8	16	3
Clarithromycin	4	8	2·0–4·0
Clofazimine	0·25	0·5	0·5–1·0
Ethambutol	4	8	5
Rifabutin	0·5	2	0·5
Sparfloxacin	2	16	1·4

[a]Yajko, Nassos & Hadley (1987); Naik & Ruck (1989); Venkatesan (1989) and Nakamura *et al.* (1990).

number of synergistic combinations was slightly reduced for rifabutin and clofazimine, it was strongly increased for ethambutol. Moreover, it is noteworthy that when amikacin was added to the two-drug combinations, antimicrobial activity was enhanced in 14 out of 37 strains (37%), shifting from an additive effect to synergism or increasing synergism; for 5 strains (15%) synergism decreased or was an additive effect, and in 18 (47%) no variation was recorded. Newly observed or increased synergism was demonstrated mainly for the combinations clarithromycin/ amikacin/ethambutol, clarithromycin/amikacin/rifabutin (seven strains each), and clarithromycin/amikacin/clofazimine (four strains). No antagonism was observed.

Discussion

We have demonstrated that combinations of antimicrobial agents including clarithromycin and rifabutin are frequently active and sometimes strongly synergic against AIDS-related MAC strains. We also found synergism between clarithromycin plus clofazimine, and between clarithromycin plus ethambutol, in agreement with the findings of Rastogi & Labrousse (1991) and Gevaudan *et al.* (1993). When amikacin was added, synergism between clarithromycin and ethambutol was strongly enhanced, compared with the two-drug combinations of clarithromycin plus rifabutin and

Table II. FIC indices of two-drug combinations against 37 strains of *M. avium*

Combination	Cumulative % (No.) of strains with FIC index of: ≤0·25	≤0·5	0·51–2·0	≥2·0
CLA + CIP	0 (0)	8 (3)	100 (34)	100 (0)
CLA + CLO	0 (0)	38 (14)	100 (23)	100 (0)
CLA + EMB	0 (0)	16 (6)	100 (31)	100 (0)
CLA + RBT	11 (4)	54 (16)	100 (17)	100 (0)
CLA + SPAR	0 (0)	3 (1)	100 (36)	100 (0)

CIP, Ciprofloxacin; CLA, clarithromycin; CLO, clofazimine; EMB, ethambutol; RBT, rifabutin; SPAR, sparfloxacin.

Table III. FIC indices of three-drug combinations against 37 strains of *M. avium*

Combination	Cumulative % (No.) of strains with FIC index of: ⩽0·37	⩽0·75	0·76–1·5	⩾2
AMI + CLA + CIP	3 (1)	14 (4)	100 (32)	100 (0)
AMI + CLA + CLO	8 (3)	35 (10)	100 (24)	100 (0)
AMI + CLA + EMB	3 (1)	32 (11)	100 (25)	100 (0)
AMI + CLA + RBT	22 (8)	51 (11)	100 (18)	100 (0)
AMI + CLA + SPAR	0 (0)	8 (3)	100 (34)	100 (0)

AMI, Amikacin; CIP, ciprofloxacin; CLA, clarithromycin; CLO, clofazimine; EMB, ethambutol, RBT, rifabutin, SPAR, sparfloxacin.

clarithromycin plus clofazimine. All other drug combinations tested were less effective. Sparfloxacin was less active than ciprofloxacin when combined with clarithromycin and with clarithromycin plus amikacin. Nevertheless, we believe that more strains need to be tested to determine whether sparfloxacin has any advantage over ciprofloxacin when used in combination.

Recent therapeutic trials (De Lalla *et al.*, 1992; Sullam *et al.*, 1994, Benson, 1994*b*) suggest clarithromycin, ethambutol and rifabutin to be the most active drugs against MAC bacteraemia. Our in-vitro data are supported by these results. We are planning to start a multicentre open trial to assess the clinical efficacy of an antibiotic regimen chosen based on the results of this study. Despite the considerable variability in the susceptibility tests of MAC strains, and a frequent lack of correlation between the results of in-vitro tests and clinical outcome, we conclude that clarithromycin potentiates the antibacterial activity of rifabutin, clofazimine and ethambutol. Clarithromycin should therefore be regarded as an essential component in effective anti-MAC chemotherapy. In our opinion, routine screening of antibiotic combinations by the radiometric system can easily be performed in microbiological laboratories and may help clinicians to optimize the therapeutic potential of drugs, and possibly reducing the emergence of resistant strains.

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(*Received 3 January 1995; returned 9 February 1995; revised 22 March 1995; accepted 5 May 1995*)