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Type Frequency and Antimicrobial Susceptibility of *Mycobacterium avium-intracellulare* Complex Strains Isolated in Italy from AIDS and Non-AIDS Patients

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Summary

Typing of the glycopeptidolipid antigens performed by thin layer chromatography on 59 *Mycobacterium avium-intracellulare* (MAC) strains isolated in Italy from AIDS patients showed that the most frequent types were 1, 4, 3, 8, and 21 (24, 19, 14, 14 and 8% of the strains, respectively). Among non-AIDS patients, types 1, 4 and 8 were also frequently found. The antimicrobial susceptibility tested in agar and/or liquid media to a panel of drugs indicated in clofazimine and rifabutin effective agents against both AIDS and non-AIDS strains. The data obtained show that MAC type distribution in Italy appears to be different from that reported for other countries. No major differences in drug susceptibility between AIDS and non-AIDS related strains were found.

Key words: Mycobacterium avium-intracellulare complex, typing, antimicrobial susceptibility.

INTRODUCTION

Organisms belonging to the *Mycobacterium avium-intracellulare* complex (MAC) have become increasingly important in human pathology [1,2]. Before the acquired immunodeficiency syndrome (AIDS) epidemic, this group of organisms was isolated mainly from old patients with chronic respiratory diseases, and disseminated infections with MAC were extremely rare [3]. MAC organisms are now considered to be the most important cause of disseminated bacterial infections complicating AIDS [4-7], with an estimated incidence of bacteremia of 43% in the two years following AIDS diagnosis [8].

Typing procedures such as seroagglutination and thin layer chromatography (TLC) of the glycopeptidolipid (GPL) antigens have allowed assessment of the distribution of the 28 estab-

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lished MAC serotypes [9-11]. In the United States [11,12] and Australia [13] the rank order of MAC isolation frequency from AIDS patients was serotype 4 > serotype 8 > serotype 1. Among European countries serotype 6 is prevalent in Sweden [14], serotypes 4 and 6 in Denmark [15] and serotype 8 in Germany [16]. These results were similar to the type pattern of isolates from non-AIDS patients [11, 13-15].

Since little is known in Italy about MAC infections in AIDS [17] and non-AIDS patients, we collected strains from patients in different clinical settings and investigated the type distribution and antimicrobial susceptibility *in vitro*.

PATIENTS AND METHODS

Mycobacterial strains and general information on the patients

Clinical isolates of MAC were collected from four hospitals or University-based Mycobacteriology Laboratories (Ancona, Florence, Milan and Rome). They were identified either by standard biochemical tests [18] or by commercial RNA-DNA hybridization kits for *M. avium* complex (Accuprobe, Gen Probe, San Diego, CA, USA). Microorganisms were maintained on Löwenstein-Jensen medium slants (Bio-Mérieux, Marcy l'Etoile, France) at room temperature. Both laboratory and medical records of the AIDS patients were reviewed to determine age, sex, risk category, source of MAC isolation, survival, CD4 cell count, drugs used for the therapy, and concomitant opportunistic infections at the time of MAC diagnosis. As for non-AIDS patients, only information on the body site specimen from which MAC were isolated could be collected.

Typing of MAC by TLC of GPLs

GPLs were analyzed as previously described [19,20]. Briefly, MAC cells grown in Löwenstein-Jensen medium were extracted with chloroform-methanol (2:1, by volume) for 18 h at 50°C. After centrifugation, clear extracts were treated with an equal amount of methanol containing 0.2 M NaOH for 20 min at 37°C. This mild saponification allowed the destruction of non-specific glycerides and the purification of alkali-stable lipids, mainly GPLs, i.e. the type antigens. The extracts were then acidified with acetic acid, evaporated to dryness, dissolved in methanol-chloroform (2:1) and washed with distilled water. The aqueous phase was discarded and the organic phase spotted onto duplicate silica plates and chromatographed in chloroform-methanol-water (65:25:4) and chloroform-methanol-water (80:19:1).

Air dried plates were sprayed with sulfuric acid containing 0.2 % anthrone; the type-specific GPL antigens were revealed as blue spots after brief heating of the plates. Extracts to be typed were co-chromatographed along with reference strains from the authenticated collection of serovars, kindly provided by Dr. A.Y. Tsang [19].

Antimicrobial agents

Amikacin, streptomycin, ciprofloxacin hydrochloride, isoniazid and ethambutol dihydrochloride (Sigma Chemical, St. Louis, MO, USA) were dissolved in distilled water; rifampicin (Sigma), rifapentine (Lepetit, Varese, Italy), clarithromycin (Abbott Italia, Rome), rifabutin (Farmitalia-Carlo Erba, Milan) and roxithromycin (Roussell-Pharma, Milan) were dissolved in methanol; erythromycin (Sigma) and azithromycin (Pfizer Italiana, Rome) were dissolved in ethanol; ofloxacin (Sigma-Tau, Rome) was dissolved in acetic acid; clofazimine (Ciba-Geigy, Basel, Switzerland) was dissolved in acetic acid-DMSO-distilled water (1:3:6); sparfloxacin (Rhone Poulenc Rorer, Vitry-Alfortville, France) was dissolved in NaOH 0.1 M. From the stock solutions, working solutions were made in distilled water. All other chemicals used were of the purest grade commercially available.

MIC determination by the agar dilution method

MICs (Minimum Inhibitory Concentrations) were determined by the twofold agar dilution technique using Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI, USA). Antibiotic plates with drug concentrations ranging from 64 to 0.06 μg/ml were inoculated with 10^2 to $3x10^3$ CFU (Colony Forming Units)/spot and incubated at 37°C in plastic bags for 14 days. The MIC was defined as the lowest drug concentration at which no visible growth of the

organism was observed. MICs inhibiting 50% (MIC_{50}) and 90% (MIC_{90}) of the strains tested were calculated.

MIC determination by the radiometric broth dilution method

Radiometric method was performed essentially as described by Heifets [21-22] by using 7H12 liquid medium containing ^{14}C palmitate as a carbon source. Bacterial growth resulting in the release of $^{14}CO_2$ was monitored by an automated BACTEC 460-TB apparatus (Johnston Laboratories, Towson, MD, USA). Briefly, appropriate dilutions of drugs were added in a volume of 0.1 ml to vials containing 4 ml of 7H12 medium as to obtain final concentrations ranging from 64 to 0.06 μg/ml. Each drug-containing vial was inoculated with approximately 10^4 to 10^5 CFU/ml in a volume of 0.1 ml. The bacterial growth was expressed as a numerical value called growth index (GI) ranging from 0 to 999. Two drug-free control vials, one with the same CFU number as drug-containing vials and one with a CFU number 100 times lower to represent 1% of bacterial population (10^2 to 10^3 CFU/ml), were also inoculated with microorganims. All vials were incubated at 37°C and the GI was read daily and recorded. The test was complete when the GI of the 1:100 diluted control was greater than 20 for 3 consecutive days during an 8-day period of growth, while at the same time the undiluted control reached the maximum value of the GI reading of 999 no earlier than the fourth day of growth. The MIC was considered the lowest concentration of the drug in which the GI did not exceed 50 for the duration of the test.

RESULTS

MAC isolates and general information on AIDS and non-AIDS patients

A total of 72 MAC isolates (59 from AIDS patients and 13 from non-AIDS patients) was collected. Medical records of MAC-infected AIDS patients were available for 50 cases *(Table 1).* The median age of these patients was 31 years and they were mostly men (82%) and drug abusers (58%) who developed MAC infection after a median of 31 months following the first positive test for HIV infection. Their median survival time was 6 months. The median CD4+ cell number was 29/μl (range 1-628). Each patient received multi-drug therapy (3.8 ± 1.1 drugs/patient). Amikacin, rifampicin, clofazimine, ethambutol and ciprofloxacin were used on more than 50% of the patients while clarithromycin, rifabutin and isoniazid were used in 32%, 16% and 16% of the patients, respectively. Other drugs were rarely administered. MAC-infected AIDS patients developed other opportunistic infections, the most frequent of which were due to cytomegalovirus (56% of the patients), *Candida* spp. (35%) and *Pneumocystis carinii* (35%). In non-AIDS patients MAC were isolated from pulmonary sites (7 patients), lymph nodes (5 patients) and other sources (1 patient).

Frequency of types

Fifty-nine MAC strains isolated from AIDS patients and 13 from non-AIDS patients were typed by TLC. Among AIDS patients the types most frequently isolated were type 1 (14 patients, 24%), type 4 (11 patients, 19%), type 3 (8 patients, 14%), type 8 (8 patients, 14%) and type 21 (5 patients, 8%). Types 2 and 9 (3 strains each), type 13 (2 strains) and types 6 and 7 (1 strains each) were also found. Three strains (5% of total) were a mixture of types.

Among the non-AIDS patients types 1 and 4 were found in 3 patients each and type 8 in 2 patients. Types 2, 3, 9 and 21 (1 strain each) were also found. One strain was a mixture of types.

Antimicrobial susceptibility

The antimicrobial susceptibility of 28 and 10 MAC strains from AIDS and non-AIDS patients, respectively, was tested in solid medium against 14 drugs *(Table 2).* The two groups of organisms showed a similar pattern of susceptibility with MIC_{50} and MIC_{90} values identical, or differing by one dilution only. Clofazimine, rifabutin and rifapentine were the most effective antimicrobials among the drugs tested (MIC_{90} values ≤2 μg/ml), while erythromycin, azithromycin and roxithromycin were the lowest active agents with MIC_{90} values of ≥64 μg/ml.

TABLE 1 - *General information about 50 Italian AIDS patients infected with Mycobacterium avium-intracellulare complex (MAC).*

MEDIAN AGE	31 years (range 7-58)	
SEX	Male	82%
	Female	18%
RISK CATEGORY	Drug abusers	58%
	Homosexuals	22%
	Others	20%
SOURCE OF MAC ISOLATION	Blood	74%
	Bone Marrow	7%
	Sputum	7%
	Bronchial lavage	4%
	Urine	4%
	Stool	4%
MEDIAN TIME BETWEEN HIV POSITIVITY AND MAC ISOLATION	31 months (range 3-97)	
MEDIAN SURVIVAL FOLLOWING MAC ISOLATION	6 months (range 1-24)	
MEDIAN CD4+ CELL COUNT	29/μl (range 1-628)	
DRUGS ADMINISTERED (% of the patients)	Amikacin	59%
	Clofazimine	57%
	Rifampicin	57%
	Ethambutol	54%
	Ciprofloxacin	54%
	Clarithromycin	32%
	Rifabutin	16%
	Isoniazid	16%
	Pyrazinamide	7%
	Imipenem	7%
	Streptomycin	5%
	Ofloxacin	5%
	Cycloserine	2%
	Roxithromycin	2%
INFECTION WITH OTHER OPPORTUNISTIC ORGANISMS (% of the patients)	Cytomegalovirus	56%
	Candida spp.	35%
	Pneumocystis carinii	35%
	Toxoplasma spp.	15%
	Herpes simplex	12%
	Cryptococcus	6%
	Staphylococcus/Salmonella spp	3%

When the susceptibility of the strains from AIDS patients was tested in liquid medium against some of the most currently used drugs as anti-MAC therapy, MIC_{90} values of clofazimine, rifabutin, ethambutol and ciprofloxacin were the same as in agar. Conversely, MIC_{90} values of rifampicin, clarithromycin and amikacin were one to two dilutions lower (data not shown).

DISCUSSION

Data from the Italian AIDS Control Service operating at the Istituto Superiore di Sanità, Rome, indicate that, from 1982 to the end of 1993, of 20,336 cases of AIDS, 508 were diagnosed as having concomitant disseminated or extrapulmonary infection with non-tuberculous mycobacteria, corresponding to a prevalence of

TABLE 2 - *Antimicrobial susceptibility determined in solid medium of MAC strains isolated in Italy from AIDS and non-AIDS patients.*

Drug	AIDS-strains MIC (μg/ml)			non-AIDS strains MIC (μg/ml)		
	50%	90%	Range	50%	90%	Range
Amikacin	8	32	2->64	16	32	4-32
Streptomycin	8	16	4-32	8	16	4->64
Rifampicin	8	16	<0.06-32	4	16	0.25-32
Rifapentine	1	2	0.12-2	0.5	2	0.12-4
Rifabutin	0.12	0.5	<0.06-0.5	0.06	1	<0.06-4
Ofloxacin	8	16	0.25-16	4	16	4-16
Ciprofloxacin	4	16	0.12-32	8	16	1-64
Erythromycin	>64	>64	0.25->64	>64	>64	64->64
Clarithromycin	4	16	0.12-16	8	8	2-64
Azithromycin	32	>64	8->64	64	>64	16->64
Roxithromycin	16	64	0.5->64	32	64	8->64
Isoniazid	4	8	0.5-32	4	16	2->64
Clofazimine	0.5	0.5	0.06-1	0.5	0.5	0.25-0.5
Ethambutol	8	8	2-16	8	16	4->64

2.5%. Of these, only 249 were reported as caused by MAC or *M. kansasii*.

In this study, among patients with AIDS, MAC was mostly isolated from drug abuser adult men. As reported by other authors [1,3,4], the microorganisms were mainly isolated from the blood and infection occurred as a late complication of AIDS which was diagnosed about 6 months before death at a stage in which T cell mediated immunity was severely depressed (median value of CD4+ cells/μl blood = 29). Conversely, in patients without AIDS, MAC was mainly isolated from pulmonary sites or lymph nodes.

A wide distribution of MAC types from AIDS patients was observed, with 95% of the isolates belonging to the *M. avium* group [23], in keeping with previous observations [4]. The rank order of isolation frequency (type 1 > type 4 > type 3 and 8 > type 21) appears to be slightly different from that of other countries. In particular, the predominance of type 1 and relatively high frequency of types 3 and 21, rarely found in other countries [11-16], seems to be peculiar of MAC distribution in Italy. For instance, in a large report from the US [11], types 3 and 21 were isolated only in 5 and 2 cases out of a total of 1,265 AIDS strains, respectively. To our knowledge, these two types have never been reported among European AIDS patients.

Susceptibilities of AIDS and non-AIDS strains tested by the agar method to a panel of antimicrobial agents were not dissimilar, in keeping with previous observations [22] and indicated that clofazimine and rifabutin are effective agents against both groups of isolates. In general, MIC values of the strains tested were within the same ranges as those found by other investigators [22, 24-27] and no particularly high resistance to the antimicrobial agents investigated was observed. No correlation between antimicrobial susceptibility and types was found (results not shown). MICs of AIDS isolates versus some clinically used antimicrobials *(Table 1)* were lower in broth than in agar, probably because the drugs, owing to the shorter incubation time of the test in liquid medium, were less degraded. These observations emphasize the need of using the broth method by the automated apparatus BACTEC as the method of choice for testing antimicrobial agents, as validated by inter- and intra-laboratory studies [28].

Our results confirm that, independently of the types found, there are no major differences in MAC susceptibility to antimicrobial agents in AIDS and non-AIDS patients. Of the drugs that were empirically administered in combination therapy to the patients from whom the

MAC studied here were isolated, rifabutin is not so widely used as could be expected on the basis of *in vitro* susceptibility of the strains tested.

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