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Rapid Susceptibility Testing for Nontuberculosis Mycobacteria Using Flow Cytometry

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We demonstrated previously that susceptibility testing of *Mycobacterium tuberculosis* could be accomplished within 24 h after the organisms were incubated with antituberculosis agents by using fluorescein diacetate (FDA) staining and flow cytometry. Continued studies have now shown that assay suspensions containing *M. avium*, *M. fortuitum*, *M. gordonae*, or *M. marinum* incubated with various concentrations of ciprofloxacin, clarithromycin, erythromycin, kanamycin, rifampin, and tobramycin hydrolyzed less FDA than drug-free controls. Suspensions of treated and nontreated mycobacteria could be easily differentiated at 6 and 24 h after the initiation of the susceptibility assays by using FDA staining and flow cytometry. In addition, multiplication of the mycobacteria was not required to discern differences between drug-free suspensions of mycobacteria and those treated with antimycobacterial agents. The flow cytometric assay is simple, reproducible, and rapid.

Recently, we showed (12) that susceptibility testing of *Mycobacterium tuberculosis* H37Ra could be performed rapidly by using flow cytometry. Results of tests were available within 24 h after *M. tuberculosis* organisms were incubated with antimycobacterial agents. The method is based on the ability of fluorescein diacetate (FDA) to pass through the hydrophobic cell membrane of viable mycobacteria and be rapidly hydrolyzed to free fluorescein by intrinsic esterase. As the fluorescein accumulates in the bacterial cells, the fluorescent mycobacteria can be easily detected by flow cytometric analysis. In contrast, dead mycobacteria or mycobacteria inhibited by antimycobacterial agents hydrolyze significantly less FDA.

The use of flow cytometry and FDA staining of mycobacteria shows considerable promise as a rapid method for obtaining test results indicating resistance or susceptibility. Conventional methods of susceptibility testing generally require 1 to 3 weeks of incubation before results are available (3, 10, 11). Although the BACTEC TB-460 system has greatly reduced the period of incubation before results are attainable, 4 to 12 days of incubation are still required (6, 8, 18, 20, 21). Even the luciferase reporter phage assay (7) and the gel microdrop encapsulation method, which employs flow cytometry (19), requires several days of incubation before results are available. In contrast, our assay can be completed within 6 or 24 h of the initiation of testing and does not require multiplication of mycobacteria.

In the present study, further evidence that flow cytometry is valuable for performing susceptibility testing of mycobacteria is provided. Nontuberculosis mycobacteria including *M. avium*, *M. fortuitum*, *M. gordonae*, and *M. marinum* were incubated with various concentrations of antimycobacterial agents. The results of susceptibility tests were available 6 to 24 h after the initiation of testing.

MATERIALS AND METHODS

Antimycobacterial agents. Ciprofloxacin (CIP) and clarithromycin (CLR) were obtained from Miles Pharmaceuticals, West Haven, Conn., and Abbott Laboratories, Abbott Park, Ill., respectively. Erythromycin (ERY), kanamycin (KAN), rifampin (RIF), and tobramycin (TOB) were obtained from Sigma Chemical Co. St. Louis, Mo. Stock solutions of CIP, CLR, ERY, KAN, RIF, and TOB were prepared at 1,280 μg/ml in distilled water, sterilized by filtration with a 0.22-μm-pore-size filter apparatus (Nalgene Lab Ware Division, Rochester, N.Y.), dispensed in 1-ml aliquots, and frozen at −70°C until use. RIF was prepared at 1,280 μg/ml in 10% methanol.

Bacteria and inoculum preparation. *M. fortuitum* ATCC 14467, *M. avium* TMC 706, *M. gordonae* TMC 1324, and *M. marinum* TMC 1218 were obtained from the American Type Culture Collection, Rockville, Md. The bacteria were grown separately in 50 ml of Middlebrook and Cohn 7H9 broth (Difco, Detroit, Mich.) at 37°C until the turbidity of the suspension was equivalent to that of a McFarland 1 standard (3×10^8 CFU/ml). This took approximately 5 days of incubation. One-milliliter samples were dispensed into 1.5-ml screw-cap tubes (Starstedt, Newton, N.C.) containing 0.5 ml of sterile glycerol (Sigma), sealed, and stored at −70°C. When needed, a frozen suspension of mycobacteria was thawed and an aliquot of 20 μl was used to inoculate fresh 7H9 broth. The culture was incubated at 37°C for 2 days before an aliquot of 20 μl was transferred to 3 ml of 7H9 broth that was incubated for an additional 3 days. Subsequently, the culture was centrifuged (IEC, Needham Heights, Mass.) at 500 rpm for 2 min to removed large clumps of bacteria. The culture was then adjusted to a turbidity equivalent to that of a McFarland 0.5 standard by the addition of 7H9 broth. Only log-phase cultures were used for susceptibility testing.

Susceptibility tests. The agar dilution method recommended by the National Committee for Clinical Laboratory Standards (M24-P) (11) was used for the determination of the MICs for *M. fortuitum*. The MIC endpoint was the lowest concentration of the antimycobacterial agent that completely inhibited visible growth (10^6 organisms) on Middlebrook and Cohn 7H10 agar medium. The MICs were determined after 3 to 4 days of incubation at 37°C. The MICs were also read 7 days later for confirmation. Prior to incubation, the plates were sealed in plastic bags to reduce drying.

A broth macrodilution test was also performed with 10^6 mycobacteria for determination of MICs and the MBCs for *M. avium*, *M. fortuitum*, *M. gordonae*, and *M. marinum*. The macrodilution method was similar to that recommended by the National Committee for Clinical Laboratory Standards (M7-A3). The MIC endpoint was the lowest concentration of the antimycobacterial agent that completely inhibited visible growth. The MICs were determined after 5 days of incubation at 37°C. The MBC was determined 5 days after incubation at 37°C by subculturing 0.1 ml of the test suspensions onto drug-free Middlebrook and Cohn 7H10 agar medium. The plates were then streaked for isolation and were incubated at 37°C for 10 days.

Preparation of assay suspensions for flow cytometric analysis and determination of viability. Serial twofold dilutions (0.5 ml) of the antimycobacterial agents were prepared with 7H9 broth in 12-by-75-mm round-bottom Falcon tubes (no. 2054; Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). Each dilution was then inoculated with 0.5 ml of 7.5×10^5 mycobacterial organisms. Drug-free suspensions of mycobacteria were also included as controls. The tubes were incubated for 6 or 24 h at 37°C before the test suspensions were analyzed by flow cytometry.

The numbers of viable mycobacteria in the drug-free controls and in the various concentrations of CIP, CLR, ERY, KAN, RIF, and TOB were determined 24 h after inoculation. Briefly, serial 10-fold dilutions of each concentra-

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tion of CIP, CLR, ERY, KAN, RIF, and TOB were prepared with 7H9 broth, and 50-μl samples were plated to determine the number of CFU per milliliter on 7H10 medium. The plates were incubated at 37°C for 10 days before the number of CFU of mycobacteria per milliliter was determined. This method was also used for determination of the numbers of viable mycobacteria in the drug-free controls.

Flow cytometric data acquisition. After incubation of assay suspensions for 6 or 24 h, 10 μl of FDA (10 μg/ml; Sigma) was added to the suspensions to yield a final concentration of 100 ng/ml. The samples were incubated at 37°C for 30 min before being analyzed with a single argon laser tuned at 488 nm (FACScan flow cytometer; Becton Dickinson Immunocytometry Systems) by using FAC Scan Lysys II software for data acquisition and analysis. Initially, viable and heat-killed *M. fortuitum* organisms were detected and differentiated from 7H9 particles by using forward scatter, side scatter, and FDA fluorescence. Live gating was performed on profiles of mycobacteria during data acquisition to exclude all 7H9 particles. Data were acquired over a period of between 7 and 60 s to count 10,000 bacteria or events.

Flow cytometric statistical analysis. Samples were analyzed by examining histogram profiles of FDA fluorescence with FACScan Lysys II software. Two parameters were evaluated: the number of events per second (number of labeled mycobacteria) and the mean channel fluorescence (intensity of fluorescence-labeled mycobacteria). These values were obtained as part of the flow cytometric statistical analysis provided with the FACScan Lysys II software.

Statistics. The values obtained were tested by analysis of variance. The Fisher least-significant-difference test was used to examine pairs of means when a significant *F* value indicated reliable mean differences (22). The alpha level was set at 0.05 before the experiments were started.

RESULTS

Detection of *M. fortuitum* by flow cytometry. Initially, data were acquired to identify viable *M. fortuitum* organisms in Middlebrook and Cohn 7H9 broth (Fig. 1A and B). Although background particles (less than two events per second) were detected in 7H9 broth (Fig. 1A), the number of particles did not significantly affect our ability to detect *M. fortuitum* organisms (Fig. 1B). When viable (Fig. 1C) and heat-killed (Fig. 1D) *M. fortuitum* organisms were incubated with FDA, only viable *M. fortuitum* organisms hydrolyzed FDA (Fig. 1C). The fluorescent viable *M. fortuitum* organisms had a mean channel fluorescence of 3,500 ± 500 (Fig. 1C), while the heat-killed *M. fortuitum* organisms failed to hydrolyze FDA and had a mean channel fluorescence of approximately 5 (Fig. 1D). In addition, the viabilities of the *M. fortuitum* organisms were not affected by the duration of exposure to or to various concentrations of FDA. When these experiments were repeated, similar results were obtained.

Effects of antimycobacterial agents on *M. fortuitum* organisms exposed to FDA and detection by flow cytometry. The ability of *M. fortuitum* organisms to hydrolyze FDA after incubation for 6 or 24 h in various concentrations of CIP, KAN, and RIF was determined (Fig. 2). The mean channel fluorescence decreased rapidly with increasing concentrations of CIP, KAN, and RIF. In general, the mean channel fluorescence decreased more rapidly in suspensions of *M. fortuitum* organisms exposed to CIP, KAN, and RIF for 24 h than for 6 h. The mean channel fluorescence decreased from approximately 4,000 to 500 or less after the *M. fortuitum* organisms were incubated with these antimycobacterial agents for 24 h. Similar results were obtained when the *M. fortuitum* organisms were incubated with increasing concentrations of CLR, ERY, and TOB (Fig. 3). By contrast, the mean channel fluorescence increased steadily from approximately 4,000 to 6,000 in drug-free controls.

In other experiments, the number of viable *M. fortuitum* organisms decreased rapidly with increasing concentrations of CIP, KAN, and RIF (Fig. 4). The decrease in the viability of the *M. fortuitum* organisms correlated with a decrease in the mean channel fluorescence for CIP, KAN, and RIF. The MICs were 0.15 μg/ml for CIP, 2.5 μg/ml for KAN, and 5 μg/ml for RIF. Each MIC was associated with a 50% or more decrease in the mean channel fluorescence compared with that for the drug-free control. No viable mycobacteria were recovered from the suspensions containing antimycobacterial agents when the mean channel fluorescence had decreased approximately 70% or more compared with that for the drug-free controls. The MBCs were 2.5 μg/ml for CIP, 2.5 μg/ml for KAN, and 40 μg/ml for RIF.

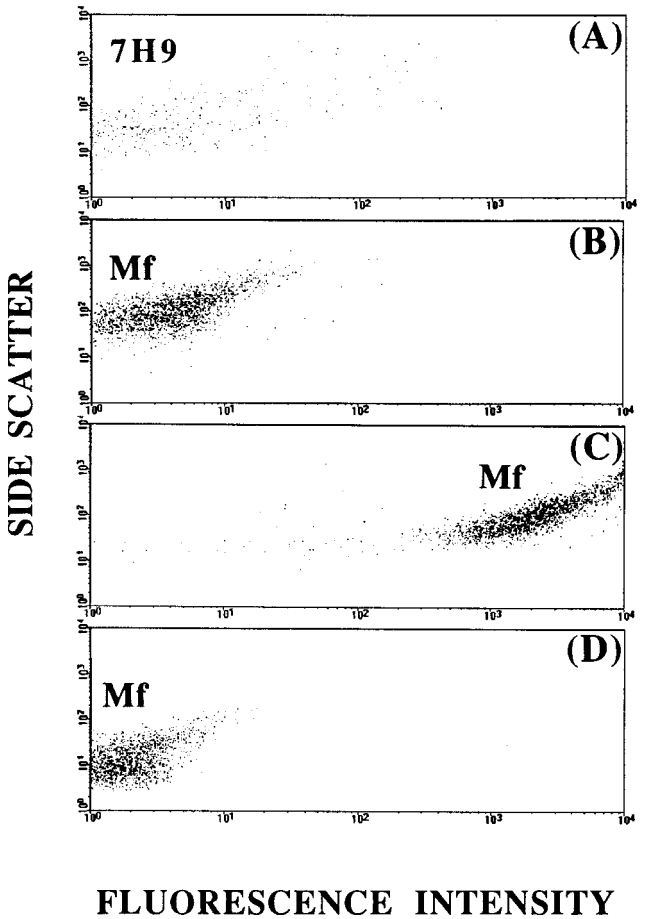

FIG. 1. Fluorescence versus side scatter of 7H9 broth (A), 7H9 broth with viable *M. fortuitum* (Mf) (B), 7H9 broth with viable FDA-labeled *M. fortuitum* (Mb) (C), and 7H9 broth with heat-killed *M. fortuitum* in the presence of FDA (D). The numbers on the *y* axis of each panel and on the *x* axis are 10^0, 10^1, 10^2, 10^3, and 10^4, respectively.

Effects of antimycobacterial agents on other mycobacterial exposed to FDA and detection by flow cytometry. Figure 5 shows the effects of various concentrations of CIP, KAN, and RIF on the ability of *M. avium*, *M. gordonae*, and *M. marinum* organisms to hydrolyze FDA and the level of detection of fluorescent mycobacteria by flow cytometry. The mean channel fluorescence decreased rapidly with increasing concentrations of the antimycobacterial agents. Similar results were obtained when *M. avium*, *M. gordonae*, and *M. marinum* organisms were incubated with various concentrations of CLR, ERY, and TOB.

DISCUSSION

The pathogenicity of *M. tuberculosis* has been clear to clinicians since its discovery by Koch in the 1890s. However, recognition of the pathogenic potential of nontuberculosis mycobacteria has lagged behind recognition of the pathogenic

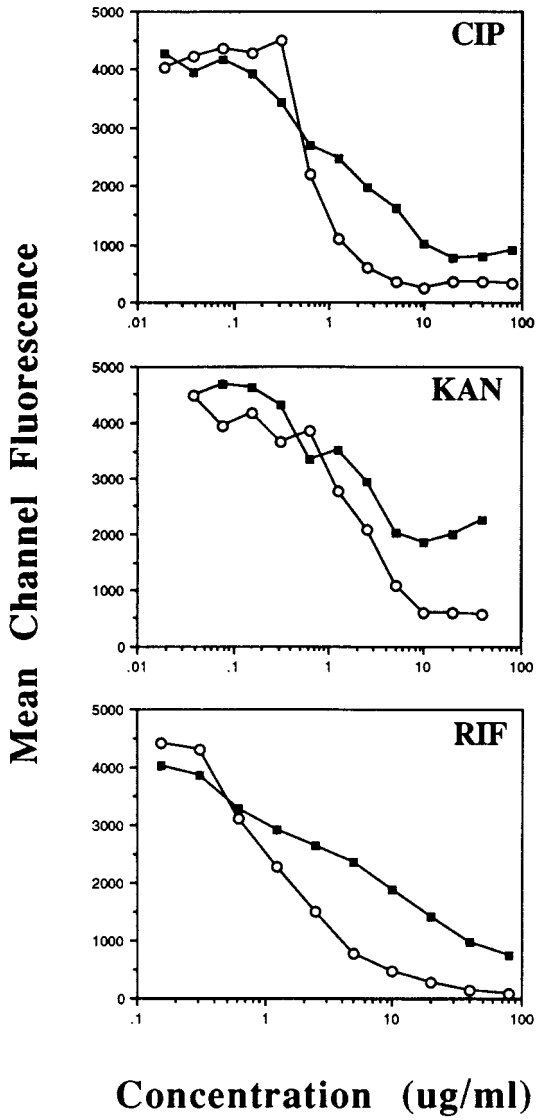

FIG. 2. Effects of antimycobacterial agents on the ability of *M. fortuitum* to hydrolyze FDA and detection of fluorescent mycobacteria (mean channel fluorescence) by flow cytometry. Assay suspensions containing *M. fortuitum* organisms with various concentrations of CIP, KAN, and RIF were incubated at 37°C for 6 h (■) or 24 h (○) before exposure to FDA and analysis by flow cytometry.

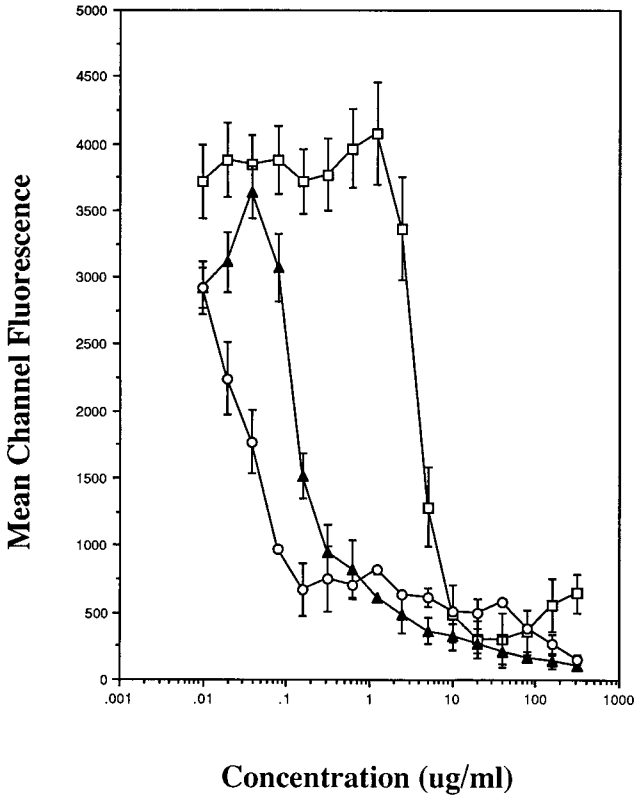

FIG. 3. Effects of CLR (○), ERY (▲), and TOB (□) on the ability of *M. fortuitum* to hydrolyze FDA and detection of fluorescent mycobacteria (mean channel fluorescence) by flow cytometry. Assay suspensions were incubated with the antimycobacterial agents for 24 h at 37°C before FDA was added prior to analysis by flow cytometry.

potential of *M. tuberculosis* (4, 13, 23). The delay in the acceptance of the pathogenic roles of these organisms arises from their relatively low level of intrinsic virulence, their propensity to commensally inhabit the airways, and their tendency to infect patients with preexisting lung disease, in which case the features of the infections caused by these organisms may be difficult to distinguish from those of the underlying disease process (13). Previously, the prevalence and distribution of nontuberculosis mycobacterial infections have been obscure (13). Recent evidence, however, suggests that the incidence of nontuberculosis mycobacterial infections, especially those caused by the *M. avium* complex, is on the rise because of the AIDS epidemic. Some hospitals and public health laboratories have reported that the overall number of cases of nontuberculosis mycobacterial infection now exceeds the number of cases of tuberculosis (4).

One must add susceptibility testing to these difficulties in recognizing the pathogenic potential of nontuberculosis mycobacteria. Conventional susceptibility testing for nontuberculosis mycobacteria is problematic (4). The clinical relevance of in vitro susceptibility testing is debated (5), even though clinicians still request them (4). The use of flow cytometry and FDA staining shows considerable promise as a rapid means of obtaining susceptibility test results to make a correlation with the clinical outcome. Flow cytometric analysis may enhance patient care by improving the designs of studies performed to assess the relevance of treatment regimens.

Our results demonstrate that susceptibility testing of nontuberculosis mycobacteria by flow cytometry can be accomplished rapidly, in 24 h or less after the initiation of testing. The technique depends on the entrance of FDA into viable mycobacteria and the conversion of FDA to fluorescein by the cellular esterases (2). In dead mycobacteria or mycobacteria incubated with antimycobacterial agents, hydrolysis of FDA fails to occur or is inhibited. The use of flow cytometry permits rapid measurement (1 min) of differences in the amounts of accumulated fluorescein between susceptible and nontreated mycobacteria. Consequently, assessment of the susceptibility and resistance of mycobacteria to various antimycobacterial agents can be performed rapidly.

The results of the current investigation extend and confirm our previous findings (12). We demonstrated previously that susceptibility testing of *M. tuberculosis* can be accomplished rapidly by using flow cytometry. Results of tests were available within 24 h after *M. tuberculosis* organisms were incubated with ethambutol, isoniazid, RIF, or streptomycin. In addition, the

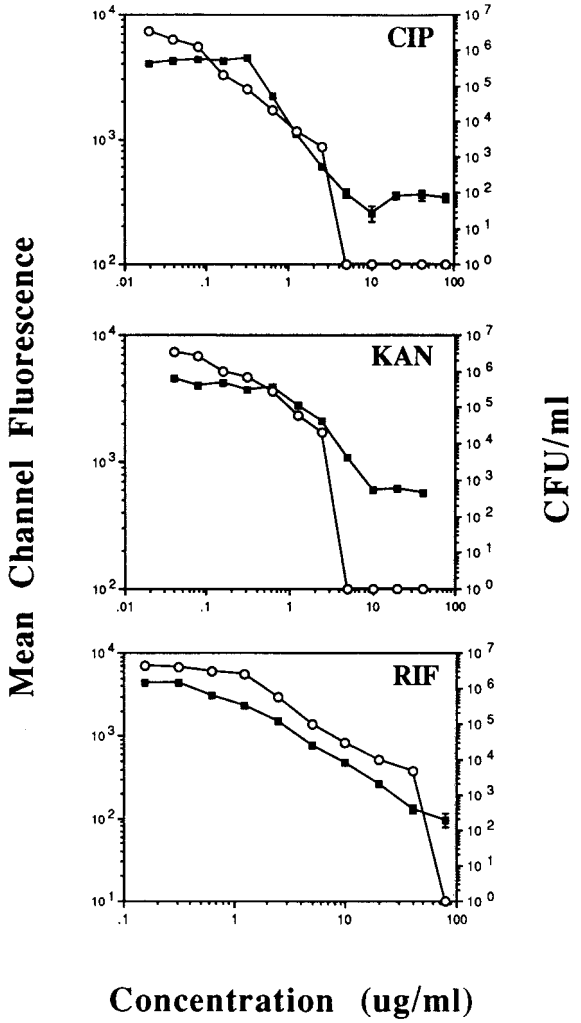

FIG. 4. Effects of various concentrations of CIP, KAN, and RIF on the viability (CFU per milliliter) of *M. fortuitum* organisms (○) and mean channel fluorescence (■). Assay suspensions containing *M. fortuitum* with various concentrations of CIP, KAN, and RIF were incubated at 37°C for 24 h before exposure to FDA and analysis by flow cytometry. Assay suspensions were also inoculated on drug-free 7H10 agar to determine the viability of the mycobacteria.

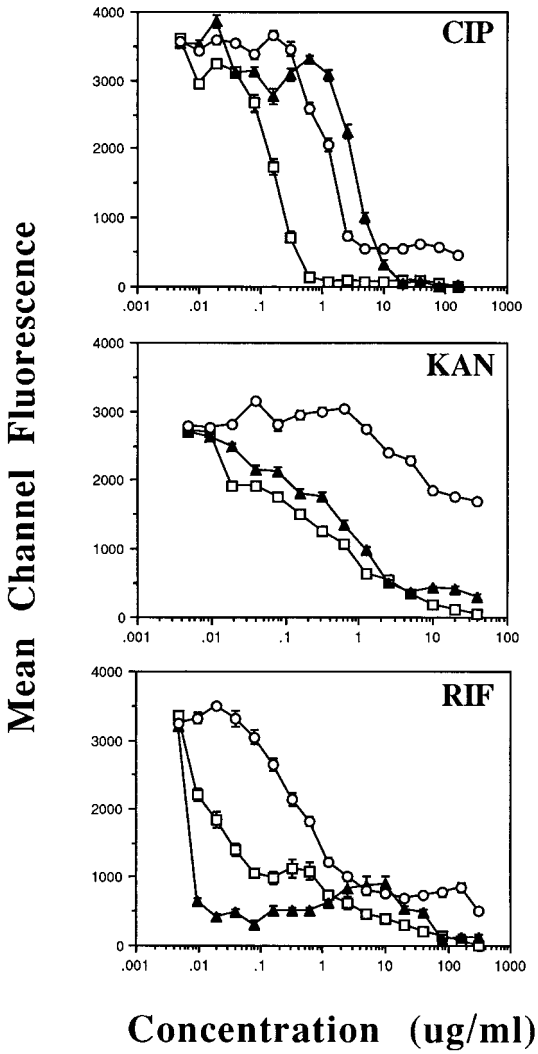

FIG. 5. Effects of various concentrations of CIP, KAN, and RIF on the ability of *M. avium* (○), *M. gordonae* (□), and *M. marinum* (▼) to hydrolyze FDA, and detection of fluorescent mycobacteria (mean channel fluorescence) by flow cytometry.

assay system did not require multiplication of mycobacteria in the drug-free control suspensions to differentiate them from drug-treated mycobacteria. In the present study, we showed that assay suspensions containing *M. avium*, *M. fortuitum*, *M. gordonae*, and *M. marinum* organisms incubated with various concentrations of CIP, CLR, ERY, KAN, RIF, and TOB hydrolyzed significantly less FDA than nontreated control organisms. The susceptibilities of treated and nontreated mycobacteria could be easily differentiated at 6 and 24 h after the initiation of the assays by using flow cytometry. Although multiplication occurred in some of the assay suspensions, multiplication was not necessary to discern differences between drug-free suspensions of mycobacteria and those treated with antimycobacterial agents.

We combined the sensitivity and detection systems of the flow cytometer with the ability of FDA to distinguish between viable and dead bacteria. The result was a more rapid method of performing susceptibility testing of mycobacteria. By measuring mean channel fluorescence (intensity of fluorescence-labeled mycobacteria), the effects of antimycobacterial agents on nontuberculosis mycobacteria and *M. tuberculosis* (12) could be determined in 24 h or less after the initiation of testing. We also suggested previously (12) that an operational definition that correlated changes in the mean channel fluorescence with the MICs of various antimycobacterial agents for mycobacteria could be developed. The MIC, as determined by flow cytometry in the present study, is that concentration of antimycobacterial agent which yielded a reduction in the mean channel fluorescence of at least 50% compared with the drug-free control value. The MICs of CIP, CLR, ERY, KAN, RIF, and TOB for *M. fortuitum* were 0.15, 0.15, 0.62, 2.5, 5, and 5 μg/μl, respectively. A decrease in the mean channel fluorescence of 50% did identify the macrodilution MIC or the MIC within one twofold dilution. Likewise, a 70% or greater decrease in the mean channel fluorescence correlated with the bactericidal concentration of each antimycobacterial agent or the MIC within one twofold dilution. Additional experiments, however, with a larger number of each *Mycobacterium* species are needed to determine accurately the percent change in

mean channel fluorescence that would predict the MIC or the MBC of each antimycobacterial agent.

Flow cytometry has increasingly been used to perform susceptibility tests for bacteria (9, 17) and yeasts (14–16). The main advantages are accuracy, reproducibility, sensitivity, objectivity, and speed. In addition, the system can be automated. A disadvantage is the identification of a suitable fluorescence indicator that accurately reflects events leading to cell inhibition or death. It is known that FDA passes through the cell membranes of viable mycobacteria (1) and other bacteria (2). Once it is inside the cytoplasm, FDA is cleaved by nonspecific intracellular esterases to form fluorescein, which causes the bacteria to fluoresce intensively. By contrast, mycobacteria treated with heat or inoculated with inhibiting concentrations of effective antimycobacterial agents cleaved less FDA and fluoresced significantly less or not at all. We found that the hydrolysis of FDA was a reliable means of assessing viability. When the mean channel fluorescence had decreased 70% or more, the mycobacteria were not viable. Frequently, the cost of a flow cytometer is also stated to be a disadvantage. This may be more perception than reality. The BACTEC TB-460 instrument is the one most frequently used for susceptibility testing. When the high cost of supplies for performing susceptibility testing with this instrument are considered, a flow cytometer will be less expensive. The reagents used with our method were relatively inexpensive. Costs were restricted to the purchase of 7H9 broth, FDA, and the antimycobacterial agents. Finally, the speed and quality of information obtained by flow cytometry increase turnaround time, which improves health care.

In conclusion, we showed that flow cytometry and FDA staining can be used to perform susceptibility testing for nontuberculosis mycobacteria. The assay is simple and reproducible and can be completed in 24 h or less from the time of the initiation of testing. The technique also does not require multiplication of mycobacteria for accurate and reproducible results.

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