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Rapid Susceptibility Testing of *Mycobacterium tuberculosis* (H37Ra) by Flow Cytometry

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The resurgence of tuberculosis has caused considerable effort to be focused on the development of rapid methods for determining the susceptibility of *Mycobacterium tuberculosis* to antimycobacterial agents. We demonstrated 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, rifampin, or streptomycin. The method was based on the ability of viable *M. tuberculosis* organisms to hydrolyze fluorescein diacetate (FDA) and the detection of fluorescent mycobacteria by flow cytometric analysis. The assay system also did not require multiplication of the mycobacteria. In contrast, *M. tuberculosis* organisms exposed to antimycobacterial agents hydrolyzed significantly less FDA. The use of flow cytometry and FDA staining shows considerable promise as a rapid method for obtaining susceptibility test results.

The incidence of tuberculosis in the United States has steadily increased since 1985 (3, 25). The resurgence of tuberculosis has caused considerable effort to be focused on the development of rapid methods for determining the susceptibility of *Mycobacterium tuberculosis* to antimycobacterial agents (6, 10, 12, 15, 19, 20). Furthermore, the recent detection of multiple-drug-resistant strains of *M. tuberculosis* (1, 3, 8, 11, 24) has made more urgent the effort to develop rapid tests to detect susceptible and resistant strains. Reducing the time required for susceptibility testing would greatly improve care of patients and control of the disease.

Conventional methods of susceptibility testing require growing *M. tuberculosis* on medium containing therapeutic agents for 2 to 3 weeks of incubation before results are obtained and reported (5, 17, 18). The most frequently used susceptibility testing method is the BACTEC TB-460 system; however, 4 to 12 days of incubation is still required before results are available (10, 16, 21–23). This method involves the measurement of $^{14}CO_2$ produced by mycobacteria growing in broth containing ^{14}C-labeled palmitic acid with or without antimycobacterial agents. To decrease further the time required for susceptibility testing, newer methods have utilized metabolic activity detected by fluorescent dyes (2, 13) or quantification of total mycobacterial rRNA hybridized to a DNA probe in the presence or absence of therapeutic agents (15). More recently, Jacobs et al. (12) described a rapid method for drug susceptibility testing by means of luciferase reporter phages. Collectively, these latter methods have dramatically reduced the time required for susceptibility testing, from weeks to days. However, none of these rapid methods, except the BACTEC system, is used routinely in clinical laboratories.

In this report, we present evidence that flow cytometry can be used to detect rapidly the susceptibility of *M. tuberculosis* to various antimycobacterial agents. Results were available within 24 h of initiation of testing, and growth of *M. tuberculosis* organisms was not required for determination of susceptibility.

MATERIALS AND METHODS

Antimycobacterial agents. Ethambutol (EMB), isoniazid (INH), rifampin (RIF), and streptomycin (STP) were obtained from Sigma Chemical Co., St. Louis, Mo. Stock solutions of EMB, INH, and STP were prepared according to the instructions of the manufacturer at 1,000 μg/ml in distilled water, sterilized by filtration with a 0.22-μm-pore-size filter apparatus (Nalgene Labware Division, Rochester, N.Y.), dispensed in 1-ml aliquots, and frozen at −70°C until used. RIF was prepared at 10,000 μg/ml in methanol.

Bacteria and preparation. *M. tuberculosis* (H37Ra, ATCC 25177) was obtained from the American Type Culture Collection, Rockville, Md. The strain was grown in 50 ml of 7H9 broth (Difco, Detroit, Mich.) at 37°C until the turbidity of the suspension was equivalent to a McFarland 1 standard (3×10^8 bacteria) at day 25 of incubation. One-milliliter samples were dispensed into 1.5-ml screw-cap tubes (Sarstedt, Newton, N.C.), sealed, and stored at −70°C. When needed, a frozen suspension of mycobacteria was thawed, and an aliquot (0.5 ml) was used to inoculate fresh 7H9 broth. The culture was incubated at 37°C for 7 days before an aliquot (0.8 ml) was transferred to 12 ml of 7H9 broth that was incubated for an additional 7 days. The culture was then adjusted by addition of 7H9 broth to a turbidity equivalent to a McFarland 1 standard. Other cultures were adjusted to contain 400 to 500 events per s by flow cytometric analysis. Only log-phase cultures of *M. tuberculosis* were used for susceptibility testing.

Agar dilution susceptibility test. An agar dilution method similar to that recommended by the National Committee for Clinical Laboratory Standards (18) was used for the determination of MICs for *M. tuberculosis*. Briefly, serial twofold dilutions of the antimycobacterial agents were prepared with 7H10 medium. The final concentrations of the therapeutic agents ranged from 0.4 to 0.0125 μg/ml for INH and RIF, 5.00 to 0.15 μg/ml for EMB, and 4.0 to 0.125 μg/ml for STP. Medium with or without antimycobacterial agents was then inoculated with 3×10^4 CFU by using a Steers replicator. Plates were incubated at 37°C and read 3 weeks after inoculation. The MIC endpoint was the lowest concentration of antimycobacterial agent that completely inhibited growth.

Preparation of assay suspensions for flow cytometric analysis and determination of viability. Serial twofold dilutions (1 ml) of the antimycobacterial agents were prepared with phosphate-buffered saline (PBS). An aliquot (0.1 ml) of each dilution of the antimycobacterial agents was then transferred to a 1.5-ml tube (Sarstedt) and inoculated with 0.9 ml containing 10^7 *M. tuberculosis* (H37Ra) organisms. The final concentrations of the therapeutic agents ranged from 0.4 to 0.0125 μg/ml for INH and RIF, 5.0 to 0.15 μg/ml for EMB, and 4.0 to 0.125 μg/ml for STP. Drug-free suspensions of *M. tuberculosis* were included as controls. The tubes were then incubated for 24 h at 37°C before aliquots were analyzed by flow cytometry. We also investigated the effect of the addition of 0.05 ml of 2.5% Formalin to the assay suspensions before analysis by flow cytometry.

The numbers of viable mycobacteria in the drug-free control suspensions and in the suspensions containing various concentrations of INH were determined 24

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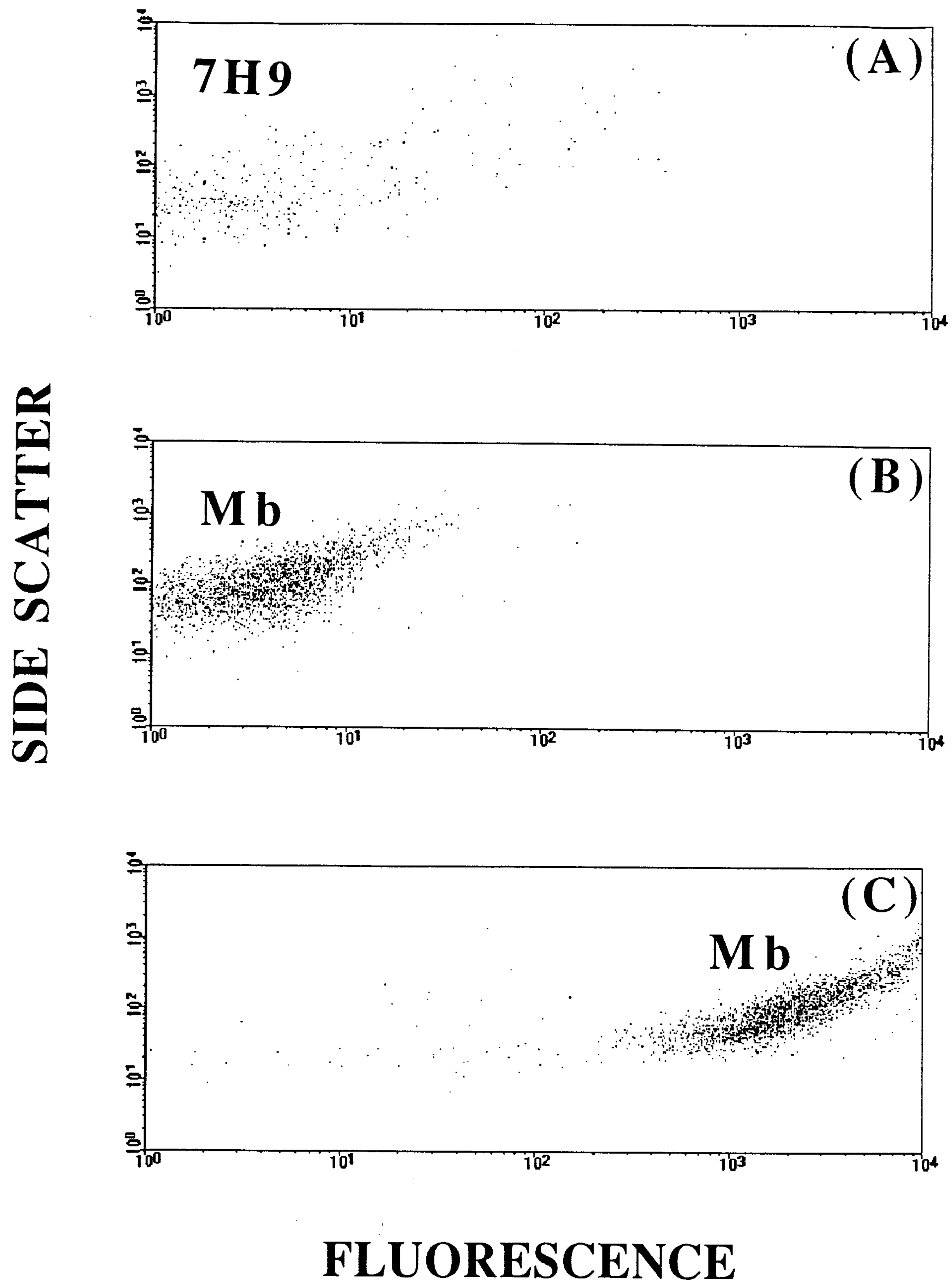

FIG. 1. Fluorescence versus side scatter of 7H9 broth (A), 7H9 broth with mycobacteria (Mb) (B), and 7H9 broth with FDA-labeled mycobacteria (C).

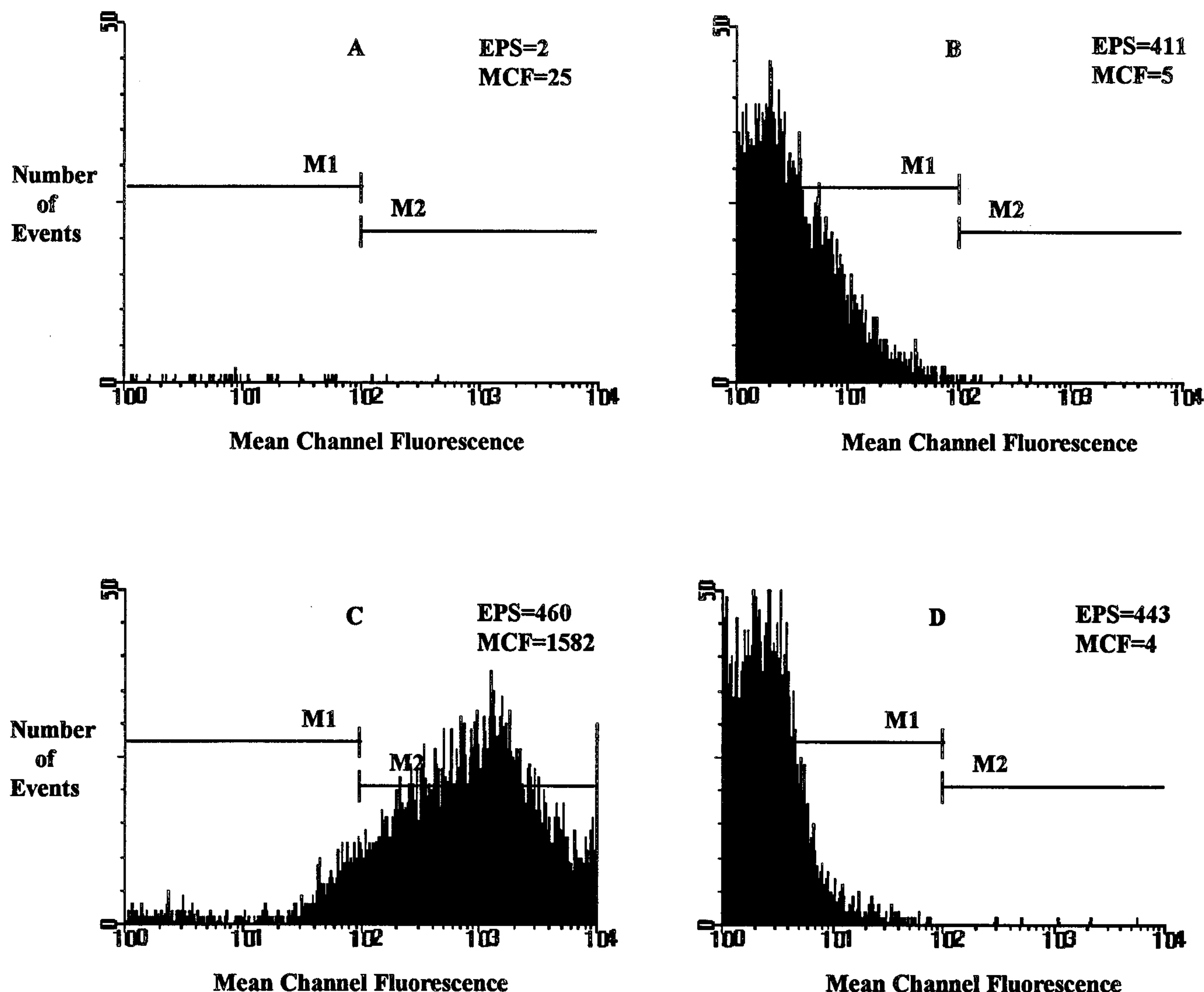

FIG. 2. Histogram profiles of 7H9 medium alone (A), with viable *M. tuberculosis* organisms (B), and with viable (C) and nonviable (D) *M. tuberculosis* organisms treated with FDA. Samples with or without 250 ng of FDA per ml were incubated at 37°C for 30 min before analysis by flow cytometry. The events per second (EPS) (number of mycobacteria) and intensity of fluorescent mycobacteria (mean channel fluorescence [MCF]) are shown.

or 48 h after incubation. Briefly, serial 10-fold dilutions of each concentration of INH were prepared with 7H9 broth, and 10-μl samples were used to determine the number of CFU per milliliter on 7H10 medium. The plates were incubated at 37°C for 3 weeks before the number of CFU of mycobacteria per milliliter was determined. Similar procedures were used for determination of the numbers viable *M. tuberculosis* organisms remaining in drug-free control suspensions and suspensions containing various concentrations of EMB, RIF, and STP.

Acquisition of flow cytometry data. After incubation of assay suspensions for 24 h, 225 μl was removed and diluted with 225 μl of PBS (pH 7.4) containing 500 ng of fluorescein diacetate (FDA) (Sigma) per ml to yield a final concentration of 250 ng of FDA per ml. The samples were incubated at 37°C for 30 min before being analyzed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) by using FACScan Lysys II software for data acquisition and analysis. Initially, viable and heat-killed *M. tuberculosis* organisms were detected and differentiated from 7H9 particles by using FDA fluorescence. Live gating was performed on profiles of *M. tuberculosis* during data acquisition to exclude all 7H9 particles (Fig. 1). In addition, calibration beads were tested daily, and the mean channel fluorescence did not vary by more than six channels. Controls also included samples containing viable and heat-killed mycobacteria in 7H9 broth. Data were acquired for 20 s to obtain approximately 10,000 counts from gated events.

Flow cytometric statistical analysis. Samples were analyzed by histogram profiles of FDA fluorescence by using FACScan Lysys II software. Gates were established for viable and heat-killed mycobacteria on the basis of their incorporation of FDA. Two parameters were evaluated: events per minute (number of labeled mycobacteria) and mean channel fluorescence (intensity of fluorescence-labeled mycobacteria). These values were obtained as part of the flow cytometric statistical analysis and were dependent upon the establishment of gates for live and heat-killed mycobacteria.

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

RESULTS

Detection of *M. tuberculosis* by flow cytometry. Viable and heat-killed *M. tuberculosis* organisms were distinguished from background particles in 7H9 medium by FDA fluorescence (Fig. 2). Few particles (<10 events per s) were detected in 7H9 medium (Fig. 2A). Viable *M. tuberculosis* organisms, however, were readily detected in 7H9 medium (Fig. 2B). When viable (Fig. 2C) and heat-killed (Fig. 2D) *M. tuberculosis* organisms were incubated with FDA, only viable *M. tuberculosis* organisms demonstrated a significant ($P < 0.01$) fluorescence inten-

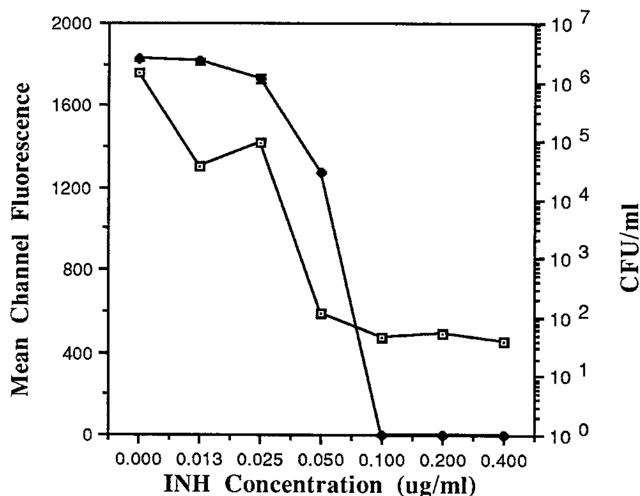

FIG. 3. Effect of various concentrations of INH on the viability of *M. tuberculosis* organisms (■) and mean channel fluorescence (□). Assay suspensions containing *M. tuberculosis* with or without various concentrations of INH were incubated at 37°C for 24 h before treatment with FDA and analysis by flow cytometry. Standard errors of the means are not shown because their small values could not be produced by the computer program.

sity, with a mean channel fluorescence of 1,582 ± 139. Heat-killed *M. tuberculosis* organisms did not hydrolyze FDA and had a mean channel fluorescence of approximately 4. When these experiments were repeated, similar results were obtained.

Kinetics of antimycobacterial activity detected by flow cytometry. The ability of *M. tuberculosis* organisms to hydrolyze FDA after incubation for 24 h in various concentrations of INH was determined (Fig. 3). The mean channel fluorescence decreased rapidly from 1,582 ± 139 for drug-free cultures of *M. tuberculosis* to approximately 500 for cultures containing 0.05 μg or more of INH per ml. When the mean channel fluorescence was determined after 48 h of incubation, similar results were obtained. The number of *M. tuberculosis* organisms (events per second), however, increased in the drug-free cultures. As a corollary, the number of viable *M. tuberculosis* organisms decreased rapidly with increasing concentrations of INH. No viable organisms were recovered when the concentration of INH was 0.1 μg/ml or more. The MIC of INH for *M. tuberculosis* determined by agar dilution was 0.05 μg/ml.

In other experiments, the mean channel fluorescence was determined after *M. tuberculosis* was incubated with various concentrations of EMB, RIF, and STP for 24 h (Fig. 4). The mean channel fluorescence rapidly decreased at concentrations of 0.31, 0.10, and 1.0 μg/ml or more for EMB, RIF, and STP, respectively. The MIC was 0.62 μg/ml for EMB, 0.05 μg/ml for RIF, and 1.0 μg/ml for STP. In addition, no viable organisms were recovered when the concentrations of EMB, RIF, and STP were 2.5, 0.2, and 2.0 μg/ml, respectively, or more. These bactericidal concentrations correlated with a mean channel fluorescence of approximately 500 or less.

When the mean channel fluorescence was determined 48 h after cultures of *M. tuberculosis* organisms were incubated in the presence of various concentrations of EMB, RIF, and STP, similar results were obtained. An increase in the number of mycobacteria (events per minute) in drug-free cultures was detected.

Effect of Formalin on mean channel fluorescence. To determine if cultures could be sterilized before analysis by flow cytometry, the effect of Formalin on mean channel fluorescence was determined. Suspensions of *M. tuberculosis* organisms were incubated with or without various concentrations of INH for 24 h (Fig. 5). FDA was added, and the assay suspensions were incubated for 30 min before Formalin was added. Flow cytometric analysis was then performed at 15, 30, 60, and 120 min after exposure to Formalin. The addition of Formalin significantly decreased the mean channel fluorescence of all assay suspensions with or without INH. Bacteria incubated in FDA for 30 min showed a slight decrease in mean channel fluorescence in the absence of Formalin. The maximum decrease in mean channel fluorescence occurred in assay suspensions treated with Formalin for 120 min. However, significant differences ($P < 0.01$) between control suspensions of *M. tuberculosis* organisms without INH and suspensions containing various concentrations of INH were still detected, particularly at concentrations of 0.025 μg/ml or more. No viable mycobacteria were recovered from assay suspensions exposed to Formalin for 60 min.

DISCUSSION

Our results demonstrate that susceptibility testing of *M. tuberculosis* can be accomplished rapidly by using flow cytometry. Results of tests were readily available within 24 h after *M. tuberculosis* organisms were incubated with antimycobacterial agents. The method was based on the ability of viable *M. tuberculosis* organisms to hydrolyze FDA to free fluorescein with detection of fluorescent mycobacteria by flow cytometric analysis. By contrast, *M. tuberculosis* organisms treated with heat or incubated with inhibitory concentrations of antimycobacterial agents hydrolyzed significantly less FDA.

It is known that FDA is a nonpolar, nonfluorescent molecule capable of diffusing across the cell walls and cell membranes of mycobacteria (2) and other bacteria (4) by active transport and passive diffusion. Once in the cytoplasm, FDA is rapidly (5 min) hydrolyzed by esterases to fluorescein. Metabolically inactive and nonviable bacteria have decreased quantities of active esterases that result in these organisms demonstrating less fluorescence (4, 13). We showed that mycobacteria susceptible to various antimycobacterial agents had diminished capacities to hydrolyze FDA. Bercovier et al. (2) also demonstrated that FDA could be used to determine the drug susceptibilities of *Mycobacterium* species.

Several rapid methods for determining the resistance or susceptibility of *M. tuberculosis* have been reported (6, 15, 16, 19). A period of 7 to 12 days, however, is required for multiplication of *M. tuberculosis* in drug-free control cultures before an assessment of the inhibitory activities of antimycobacterial agents can be made. Our assay system does not require the number of mycobacteria in the drug-free control cultures to increase. A determination of susceptibility can be made by comparing the mean channel fluorescence of mycobacteria in the drug-free culture with the mean channel fluorescence of suspensions of mycobacteria exposed to antimycobacterial agents. Results were available within 24 h of initiation of the tests. These findings are important and greatly decrease the time for obtaining susceptibility test results.

We combined the vital staining of FDA for bacteria with the ability of flow cytometry to recognize individual microorganisms. The outcome was the rapid determination of the susceptibility of *M. tuberculosis* to antimycobacterial agents. Our results showed that mycobacteria incubated in drug-free medium for 24 h and then treated with FDA were intensely fluorescent (high mean channel fluorescence) when analyzed by flow cy-

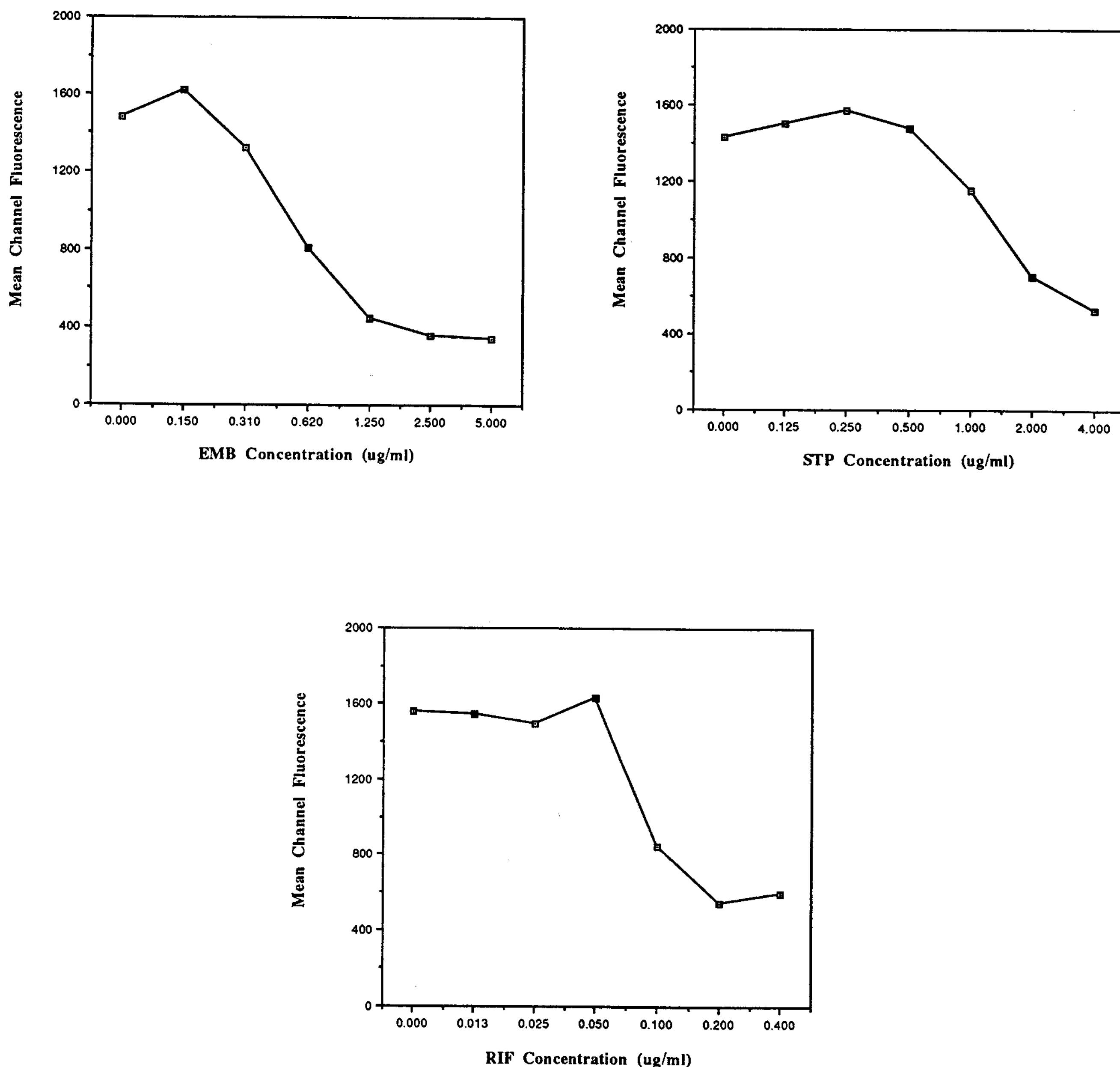

FIG. 4. Effect of various concentrations of EMB, RIF, and STP on mean channel fluorescence. Assay suspensions containing *M. tuberculosis* with or without various concentrations of antimycobacterial agents were incubated at 37°C for 24 h before treatment with FDA and analysis by flow cytometry. Standard errors of the means are not shown because their small values could not be produced by the computer program.

tometry. The mean channel fluorescence decreased rapidly when *M. tuberculosis* organisms were incubated with increasing concentrations of EMB, INH, RIF, and STP. The decrease in mean channel fluorescence correlated with a decrease in the viability of *M. tuberculosis* organisms. Bercovier et al. (2) also showed that the level of fluorescence decreased in FDA-treated *M. tuberculosis* (H37Ra) and in *Mycobacterium bovis* killed with RIF, by using a Perkin-Elmer MPF44A spectrophotofluorometer.

The MICs for our test bacterium obtained by the agar dilution (18) method were 0.62, 0.05, 0.05, and 1.0 μg/ml for EMB, INH, RIF, and STP, respectively. An operational definition of susceptibility was established to distinguish changes in mean channel fluorescence that would predict the MICs. If the mean channel fluorescence of the mycobacterial culture containing an antimycobacterial agent was 40% lower than the mean channel fluorescence obtained with the drug-free culture, the MIC was identified. For INH and EMB, a 40% decrease in mean channel fluorescence correlated with the MIC. For RIF and STP, a 40% decrease in mean channel fluorescence correlated with a twofold increase in the MIC (0.10 μg/ml for RIF and 2.0 μg/ml for STP). Thus, the MIC predicted by use of flow cytometry and FDA staining agreed with or was within one twofold dilution of the MIC determined by the agar dilution method (18). This minor increase in MIC obtained with RIF and STP by using mean channel fluorescence was within acceptable limits of one dilution. A possible explanation for the discrepancy may be that the amount of RIF and STP is inad-

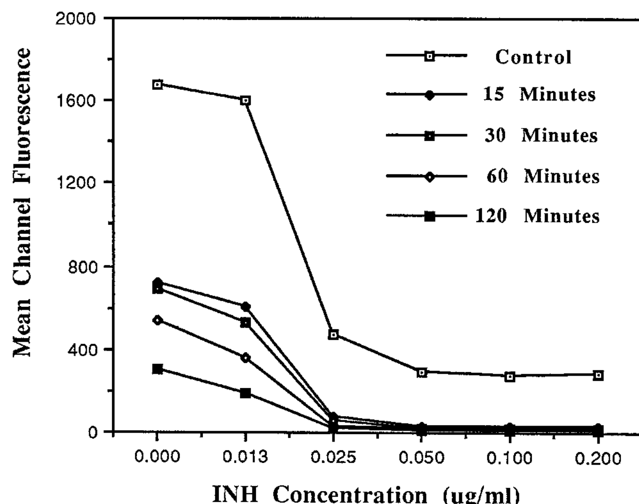

FIG. 5. Effect of duration of Formalin treatment on mean channel fluorescence of FDA-labeled *M. tuberculosis* organisms in the absence or presence of various concentrations of INH. Assay suspensions with or without INH were incubated at 37°C for 24 h, labeled with FDA, and treated with Formalin for 15, 30, 60, and 120 min before analysis by flow cytometry. Standard errors of the means are not shown because their small values could not be produced by the computer program.

equate to kill the large number of mycobacteria (10^7 CFU/ml) used in our assay suspensions. The agar dilution method used an inoculum of 10^4 CFU/ml.

The large size of the inoculum used in these flow cytometric studies, however, has a distinct benefit. Drug-resistant mutants exist in all populations of drug-susceptible strains of *M. tuberculosis* (7, 17). The larger the inoculum, the greater the chances of an assay system finding resistant organisms. The inoculum we used (10^7 CFU/ml) was large enough to detect spontaneous mutations that could introduce resistance into the population. Additional studies are needed to detect specifically these resistant mycobacteria by flow cytometry.

Our results also showed that flow cytometry and FDA staining could be used to determine the MBC of each antimycobacterial agent. The incubation of *M. tuberculosis* in 0.10 μg of INH per ml for 24 h resulted in the recovery of no viable mycobacteria. This correlated with a 70% or greater decrease in mean channel fluorescence compared with the control. Likewise, 2.5 μg of EMB per ml, 0.20 μg of RIF per ml, and 2.0 μg of STP per ml killed *M. tuberculosis*. This correlated with decreases in mean channel fluorescence of 75, 65, and 50% compared with the controls for EMB, RIF, and STP, respectively. These results showed that criteria for determining the bactericidal concentrations based on mean channel fluorescence varied with the antimycobacterial agent. Thus, mean channel fluorescence can be used to predict bactericidal activity.

The use of flow cytometry and FDA staining shows considerable promise as a rapid means to obtain susceptibility test results. Although our results were obtained with a vaccine strain (H37Ra), there is no evidence to indicate that similar results will not be obtained with virulent isolates of *M. tuberculosis* or other *Mycobacterium* species. In fact, RIF-resistant and -susceptible strains of *M. bovis* were distinguished by staining with FDA (2). Additional studies with a large number of virulent strains of *M. tuberculosis* that differ in their resistance and susceptibility to antimycobacterial agents, including multiple-drug-resistant strains, are needed. These studies will complete the determination of the utility of flow cytometry for susceptibility determinations for *M. tuberculosis.*

Flow cytometry is increasingly being used for the detection of microorganisms that are resistant or susceptible to antimicrobial agents (9, 14). In general, flow cytometric analysis is rapid because conventional susceptibility tests depend on multiplication of the microorganism for the detection of visible growth, which requires several days (12, 16, 21–23). Since multiplication is not necessary for the detection of viable and nonviable mycobacteria by our method, flow cytometric analysis is more rapid than the commonly used radiometric method (16, 21–23). Limiting factors in using flow cytometry are the cost of instrumentation, the perceived complexity of analysis, and safety. When the high cost of supplies for the radiometric instrument is considered, a flow cytometer may be less expensive. The reagents used in our method were also relatively inexpensive. Technician times for performing the radiometric method and flow cytometry, however, are similar. We also showed that Formalin treatment of assay suspensions of *M. tuberculosis* can disinfect cultures. This procedure increases safety while the flow cytometer is used, without lessening the quality of the assay. However, it is more appropriate to perform the assay in a biosafety level 3 facility, eliminating the need for the use of Formalin.

In conclusion, we showed that flow cytometry and FDA staining can be used to determine the susceptibility of *M. tuberculosis*. The assay can be completed within 24 h of initiation of testing and does not require multiplication of *M. tuberculosis*.

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