JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 5–10 Vol. 35, No. 1
0095-1137/97/$04.00+0

Rapid Susceptibility Testing of Fungi by Flow Cytometry Using Vital Staining

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Received 11 July 1996/Returned for modification 12 August 1996/Accepted 26 September 1996

A 1-h assay for antifungal susceptibility testing measuring the impairment of fungal metabolic activity was developed. Yeast viability was analyzed by flow cytometry with a novel fluorescent probe, FUN-1, which emits a red fluorescence when the yeast is metabolically active. For nine *Candida albicans* strains tested, this method yielded results comparable to those obtained by the standard M27 procedure for amphotericin B, flucytosine, fluconazole, and ketoconazole. Whether the flow cytometry antifungal susceptibility test results correlate with the in vivo activities of the drugs remains to determined.

The explosion in the rates of opportunistic fungal infections (1, 2) combined with the increasing number of reports of resistance to all the available antifungal agents (3, 5, 13, 16, 22, 26, 27) has resulted in increased interest in clinically relevant methods for antifungal susceptibility testing (10). Subsequently, results of MIC testing were shown to vary as much as 50,000-fold when a variety of disparate methods were compared (5, 14). More recently, a National Committee for Clinical Laboratory Standards method (M27-P) has been implemented (6, 7, 17, 25). Although, as it is currently configured the interlaboratory agreement for the M27 method is comparable to that for antibacterial testing (20), the relation of these in vitro data to the clinical outcome has not been determined (9).

It is a widely held view that measurements of growth inhibition and/or viability are desirable features of an antimicrobial susceptibility test. In addition to the methodological heterogeneity and the constitutive microbiological diversity within a suspension, there is the further possibility that an antifungal agent might not be evenly distributed among all of the organisms therein. Flow cytometry has been used to distinguish living from dead microorganisms by using DNA-binding vital stains (23), assess the engulfment and killing activities of phagocytic cells, and monitor the synthesis of chitin on regenerating protoplasts (10, 11, 15, 20). More recently, impermeant fluorescent dyes (ethidium bromide, propidium iodide, and fluorescein diocetate) were used to indicate antimicrobial properties (8, 14, 17, 18, 22, 25). Fluorescent staining with these dyes is based on antifungal agent-mediated membrane damage and penetration of the dye into dead cells. The length of time required for this effect ranges between 3 and 9 h (18, 19, 25).

We developed an assay using the opposite approach to assess antifungal activity: measurement of metabolic activity with the dye FUN-1. This dye is converted to a red fluorescent probe only in actively respiring fungal cells (4). The conversion requires both plasma membrane integrity and metabolic activity (26). We evaluated the potential of using this dye to measure the antifungal activities of different, nonrelated compounds with different modes of action involving the metabolic activity and/or the plasma membrane (21). Amphotericin B, flucytosine, fluconazole, and ketoconazole were tested against nine *Candida albicans* strains with different degrees of individual resistance (as determined by conventional methods) (9).

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MATERIALS AND METHODS

Culture and test strains and inoculum suspension. Six clinical isolates and the National Committee for Clinical Laboratory Standards strains *C. albicans* ATCC 26278, ATCC 90028, and ATCC 90029 subcultured on Sabouraud dextrose agar plates were used in the study.

The stock yeast inoculum suspensions were prepared as described previously (18) and were adjusted to the desired concentration by the spectrophotometric method. Briefly, each yeast was subcultured onto Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.), and inoculum suspensions were prepared for each experiment from 24-h-old cultures grown at 35°C. The turbidities of the resulting yeast suspensions were measured with a spectrophotometer at 530 mm and were adjusted to the percent transmission that matched that of a 0.5 McFarland standard. Yeasts were grown to the late log phase (usually 10^7 to 10^8 cells/ml) in yeast peptone dextrose (Difco) medium (22). The yeast culture was added to sterile water containing 2% D-(+)-glucose and 10 mM sodium HEPES (pH 7.2) in a cell culture tube. The yeast cells were centrifuged at 10,000 × *g* for 5 min at 25°C. The supernatant was removed and the pellet was resuspended in sterile water [filtered through a 2-μm-pore-size membrane containing 2% D-(+)-glucose and 10 mM sodium HEPES (pH 7.2) (test medium [TM] broth)]. A final yeast inoculum of 10^6/ml was used for both the M27 procedure and the assessment by flow cytometry.

Susceptibility tests. A total of 300 μl of the yeast suspension was added to Falcon tubes (no. 2054; Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). Stock solutions of amphotericin B (Bristol-Myers Squibb, Munich, Germany), fluconazole (Pfizer, Vienna, Austria), ketoconazole (Janssen, Beerse, Belgium), and flucytosine (Roche, Vienna, Austria) were provided by the manufacturers. Serial twofold dilutions (128 to 0.0625 mg/liter) of the antifungal agents were prepared with TM broth in Falcon tubes (Becton Dickinson). For the assay, 25 μl of the dilutions of antifungal compounds was added to 300 μl of fungi in TM broth. For controls, organisms were diluted in TM broth alone or were suspended in 70% ethanol. The tubes were incubated at 35°C with continuous shaking for 30 min. Thereafter, FUN-1 (Molecular Probes, Eugene, Oreg.) was added at a final concentration of 5 μmol. Thereafter, the yeast cell suspension (with or without antifungal agents) was incubated for an additional 30 min at 35°C in shaking water bath.

Flow cytometry data acquisition. The cells were analyzed with a single argon laser tuned at 488 nm (FACScan Flowcytometer; Becton Dickinson Immunocytometry Systems) by using FACScan Lysis II software for data acquisition and analysis. Initially, the cells were detected and differentiated from the background fluorescence by dot blot analysis of the forward and side scatters (see Fig. 1A). A total of 10,000 fungi were acquired for analysis.

Flow cytometry statistical analysis. The samples were analyzed by using histogram profiles of FUN-1 fluorescence, as indicated in Fig. 1 to 4. The mean channel fluorescence (intensity of fluorescence-labeled fungi) between control and drug-treated suspensions was compared in FL 2 (i.e., red fluorescence detection of 560 ± 15 nm). The values were obtained as part of the flow cytometry statistical analysis provided with FACScan lysis II software. When drug-treated cells were analyzed, impairment of cell metabolism and respiration was evidenced by decreased FUN-1 staining. Damaged fungi, such as those incubated in 70% ethanol, moved to the left on the *x* axis (see Fig. 3 and 4). The minimal fungicidal concentration (MFC) was the lowest concentration of drug in

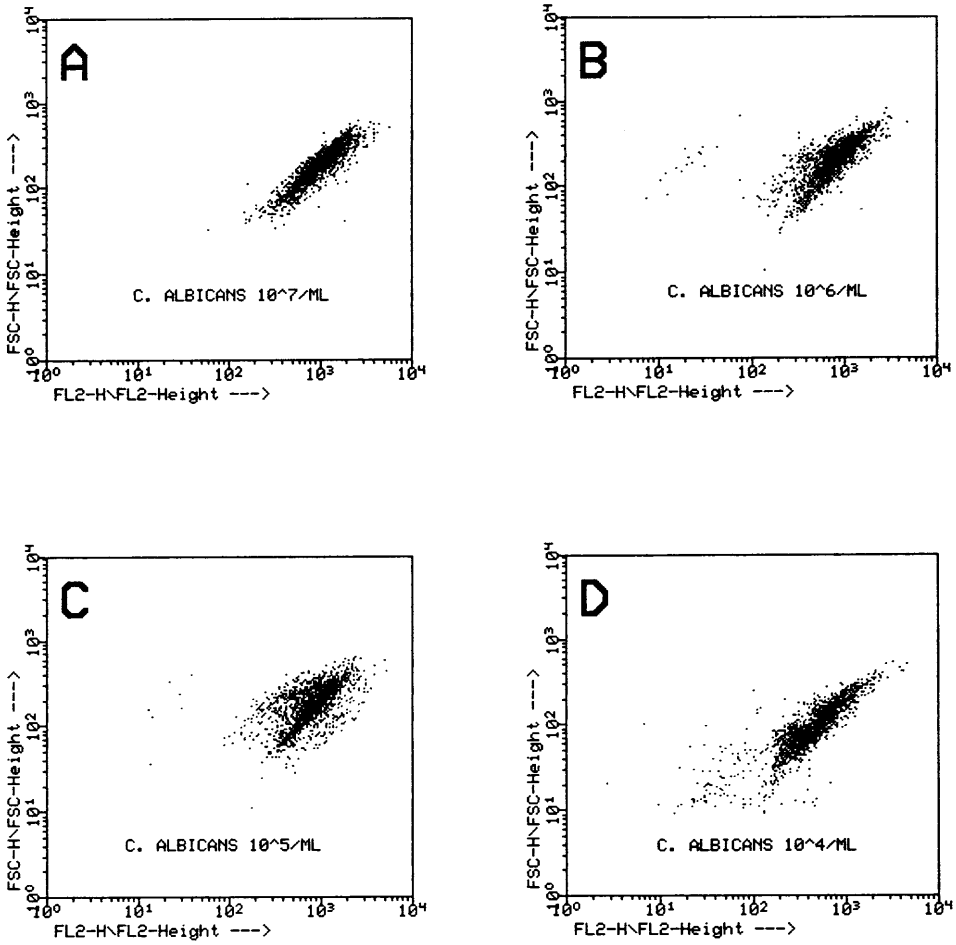

FIG. 1. Effect of different *C. albicans* concentrations on flow cytometry data acquisition and depiction with 5 μM FUN-1. Typical dot blots with forward scatter and FL2 signals are shown: (A) *C. albicans* at 10^7/ml; (B) *C. albicans* at 10^6/ml; (C) *C. albicans* at 10^5/ml; (D) *C. albicans* at 10^4/ml.

the dilution series that did not produce a decrease in the fluorescence of FUN-1 compared with that at the next higher concentration (lowest mean channel fluorescence in FL 2). In other words, the MFC is the lowest concentration at which maximal suppression of FUN-1 fluorescence is observed. The MIC was defined as the lowest drug concentration in the dilution series that produced a reduction in FUN-1 fluorescence (reduction of the mean fluorescence channel in FL 2).

Conventional susceptibility testing. The MICs of the antifungal agents were determined by the broth macrodilution method by the M27 procedure (9). All strains were tested by using a standardized inoculum of 10^4/ml in RPMI 1640 with L-glutamine and morpholinepropanesulfonic acid (0.165 M; Difco).

RESULTS

Effects of different FUN-1 and yeast cell concentrations. Yeast cell concentrations of 10^4 to 10^7/ml were tested (Fig. 1). FUN-1 fluorescence was tested in the range of 5 to 20 μM (Fig.

TABLE 1. MICs of amphotericin B, flucytosine, fluconacole, and ketoconazole for nine *C. albicans* strains determined by flow cytometry and the M27 procedure

C. albicans strain	MIC (mg/liter)								
	Amphothericin B		Flucytosine			Fluconazole		Ketoconazole	
	M27 procedure	Flow cytometry	M27 procedure	Flow cytometry, 0.5 h	Flow cytometry 5 h	M27 procedure	Flow cytometry	M27 procedure	Flow cytometry
4120	0.125	0.125	0.12	>128	0.25	4	0.125	16	16
4141	0.25	0.063	0.12	>128	0.25	128	128	64	32
3953	0.25	0.063	0.12	>128	0.25	128	128	32	16
3977	0.125	0.063	0.12	>128	0.25	128	>128	32	16
4184	0.25	0.063	0.28	>128	0.25	128	>128	16	8
4198	0.125	0.063	0.25	>128	0.125	128	>128	16	16
ATCC 90028	0.25	0.063	1	>128	2	1	0.5	0.5	0.125
ATCC 90029	0.25	0.063	>128	>128	>128	0.5	0.25	0.25	0.063
ATCC 26278	0.25	0.063	1	>128	4	32	2	128	64

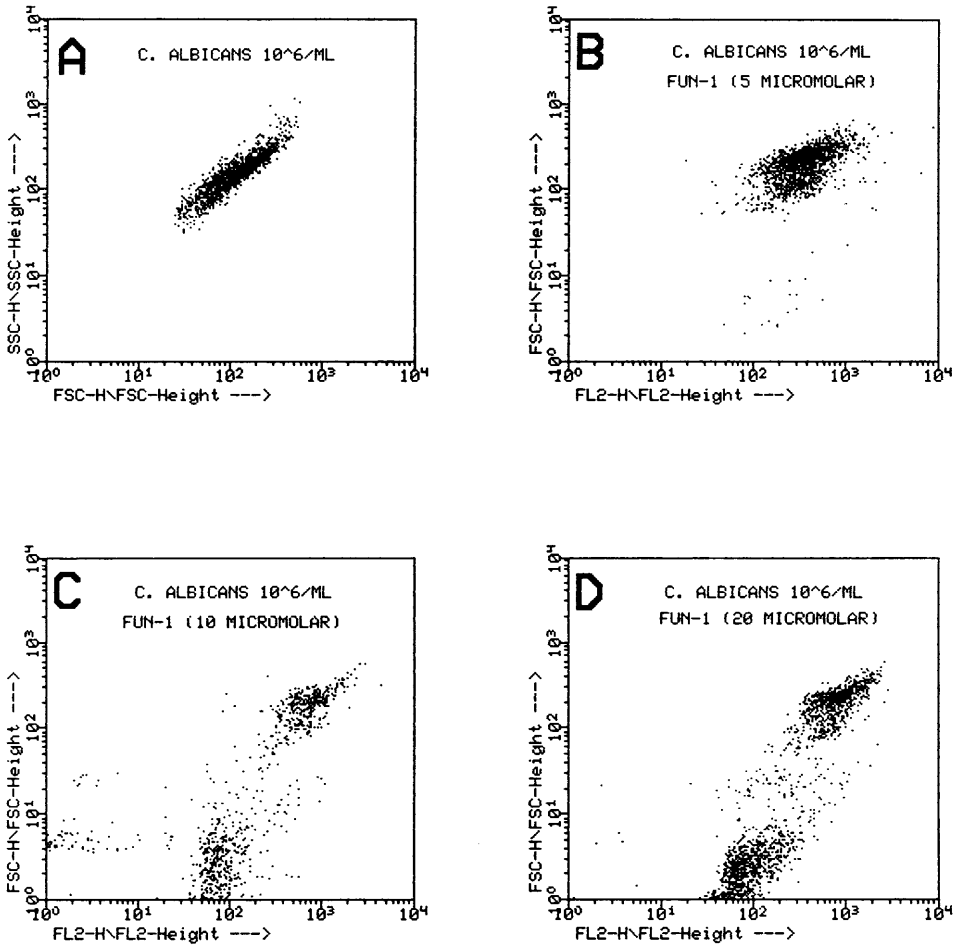

FIG. 2. Effect of different FUN-1 concentrations on fluorescence emission by using *C. albicans* at a concentration of 10^6/ml. (A) Typical dot blot showing forward (FSC) and sideward (SSC) scatter signals of *C. albicans* without FUN-1 staining. (B) FUN-1 at 5 μM. (C) FUN-1 at 10 μM. (D) FUN-1 at 20 μM.

2). A constant mean fluorescent emission was seen with 5 μM FUN-1 at a yeast concentration ranging from 10^5 to 10^7/ml (Fig. 1A to C). Lower concentrations not only extensively prolonged the acquisition time but also increased the background fluorescence (Fig. 1D). In experiments with 5 to 20 μM FUN-1 for the staining of the yeast suspensions, a concentration of 10 and 20 μM FUN-1 yielded a higher background fluorescence (Fig. 2C and D), and optimal staining was seen with 5 μM FUN-1 (Fig. 2B). Figure 2A depicts a typical dot blot analysis of the forward and sideward scatters of viable *C. albicans* organisms.

The effect of drug treatment for 30 min on FUN-1 staining is depicted in Fig. 3 and 4. The impairment of FUN-1 staining was related to drug concentration for samples treated with amphotericin B, fluconazole (Fig. 4A and B), and ketoconazole (Fig. 4C and D). In contrast, treatment of fungal cells with flucytosine did not alter FUN-1 staining after 30 min of incubation. After 5 h of incubation, however, FUN-1 staining was also diminished in a dose-dependent manner. Figure 5 depicts the flow cytometry determination of the MIC and MFC of amphotericin B for *C. albicans* ATCC 90029.

A comparison of the data obtained by flow cytometry and the conventional technique is presented in Table 1. Testing of amphotericin B by the M27 method and flow cytometry yielded corresponding results. After 5 h of incubation with flucytosine, the flow cytometry and M27 methods yielded similar MICs. In addition, a correlation between the M27 procedure and the assessment by flow cytometry with respect to fluconazole resistance testing was found. Similar results were found for ketoconazole. A relationship was found between the MICs obtained by the flow cytometry and M27 methods. The MFCs are depicted in Table 2.

DISCUSSION

Antimicrobial susceptibility testing should be easy to perform and cost-effective and should provide MIC endpoints which are reproducible and readily determined after a short incubation period. In addition, these MIC results should serve as predictors of drug efficacy or in vivo response. The currently available standardized and nonstandardized methods were recently demonstrated not to correlate with clinical outcome (9). Determination of the clinical relevance of these methods and interpretive breakpoints was not possible.

Flow cytometry has increasingly been used to perform susceptibility tests for bacteria (19, 22) and yeasts (18, 19, 23, 25, 26). Previous approaches, however, focused on dyes which penetrated only damaged cells with membrane defects. The length of time required for the membrane to be damaged is an important consideration. In the case of an organism such as *C.*

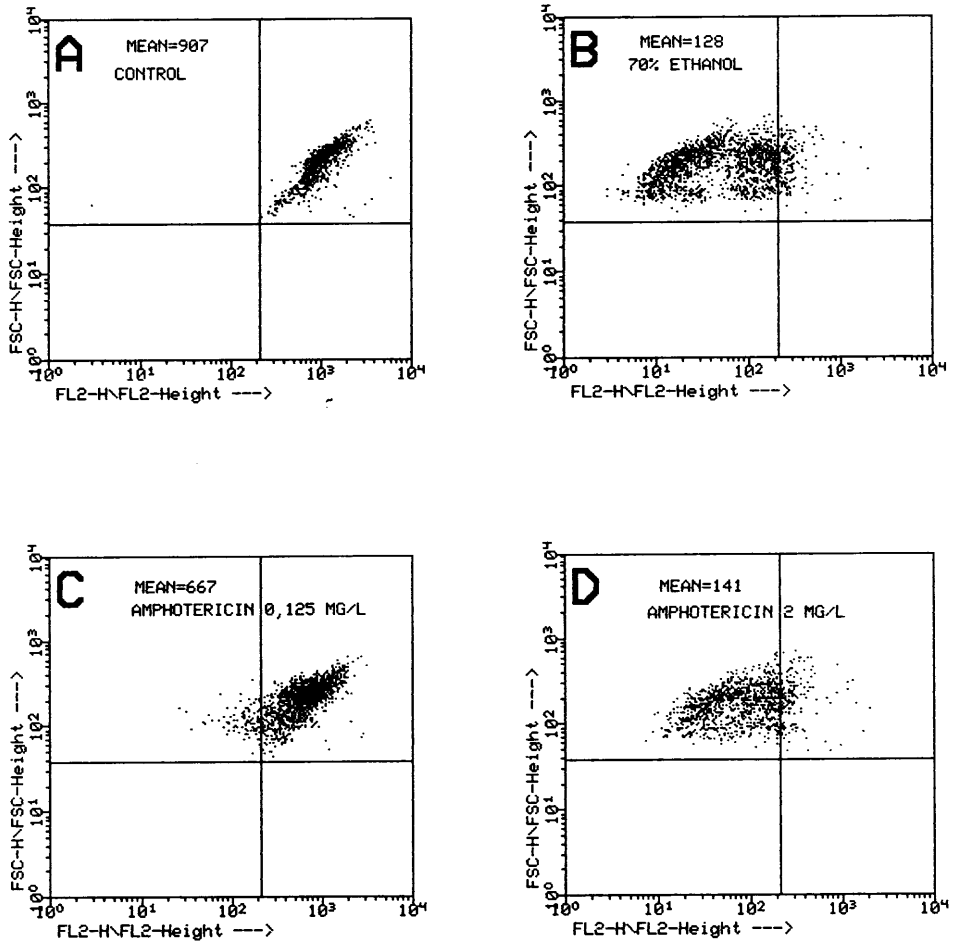

FIG. 3. Effect of 70% ethanol and amphotericin B on FUN-1 staining of *C. albicans*. Shift of the mean FL 2 signal to the right indicates impaired staining compared with that for the control. (A) Control dot plot (forward scatter and FL2) without ethanol or antifungal agents; (B) 70% ethanol; (C) 0.125 mg of amphotericin B per liter; (D) 2 mg of amphotericin B per liter.

albicans, with a generation time of approximately 3 h, membrane damage was detected after 5 h, although a 9-h incubation period leads to the optimal separation of damaged and undamaged cells (21). In another study (18), a 7-h period of incubation was used, while a third study (25) found that the exposure to the antibiotic for a 3-h period was all that was required. In our assay, membrane damage was not a requirement for the impairment of fluorochrome staining. In contrast, early drug-mediated impairment (30 min) of fungal metabolic and respiratory function could be detected with FUN-1. Since this dye is also affected by membrane damage, the antifungal activities of compounds with different modes of action can be measured. An incubation period of 30 min, however, is too short to detect the fungistatic effect of flucytosine. This corresponds to its mode of action, requiring the incorporation of deaminated flucytosine into fungal RNA. However, after an incubation period of 5 h, similar effects of FUN-1 staining were seen (Table 2).

The flow cytometry assay demonstrated a good correlation with the standard M27 procedure for assessing MICs. However, with regard to yeast infections, patients respond to treatment with azoles, even though the concentrations of these

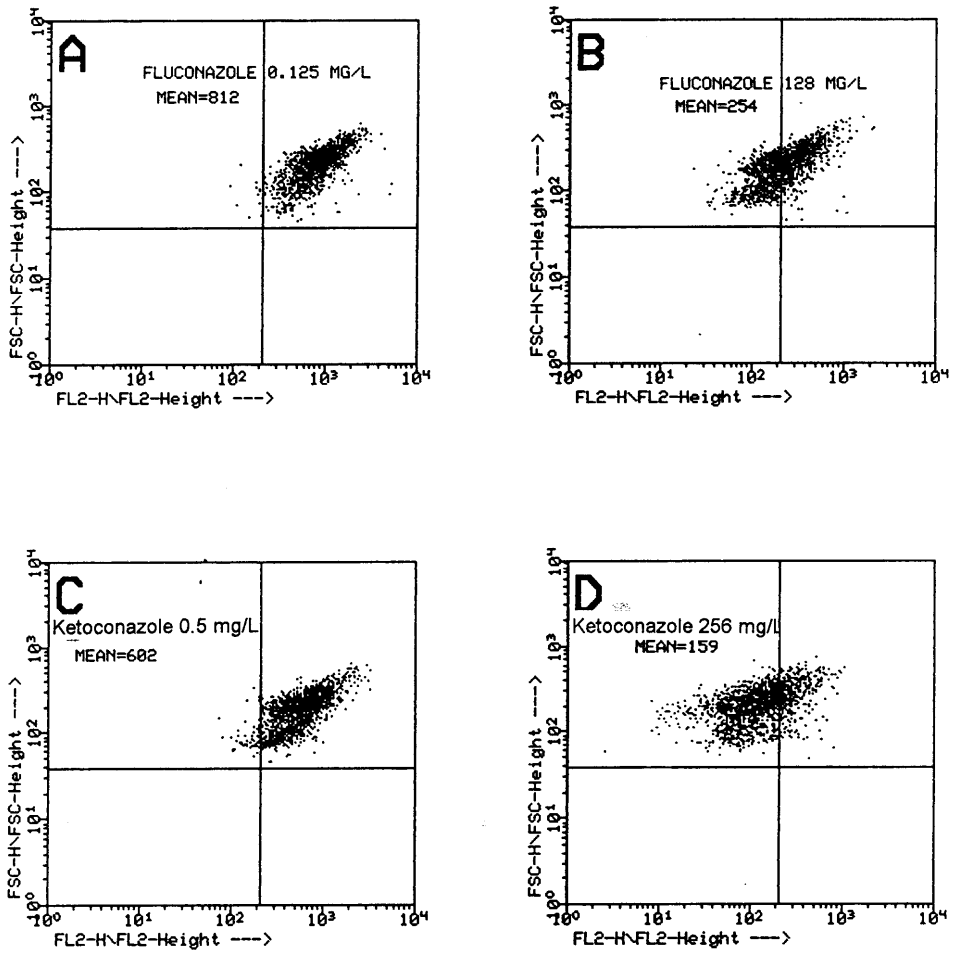

FIG. 4. Effect of fluconazole and ketoconazole on FUN-1 staining of *C. albicans*. Shift of the mean FL 2 signal to the right indicates impaired staining compared with that for the control. (A) Fluconazole at 0.125 mg/liter; (B) fluconazole at 128 mg/liter; (C) Ketoconazole at 0.5 mg/liter; (D) ketoconazole at 256 mg/liter.

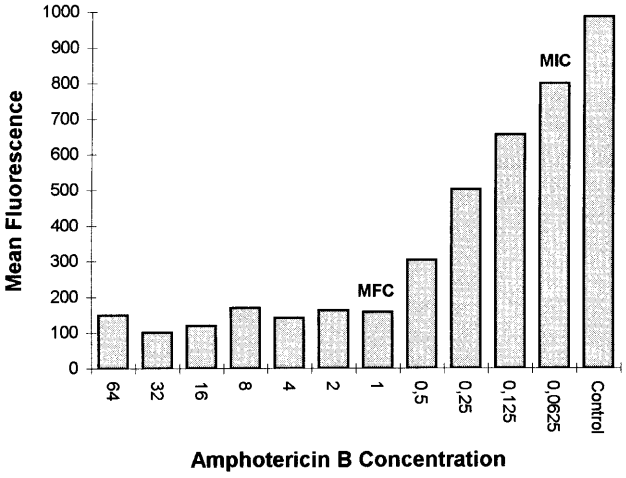

FIG. 5. Assessment of MIC (lowest drug concentration that produces a reduction in FUN-1 mean fluorescence) and MFC (lowest concentration with maximal suppression of FUN-1 fluorescence) by flow cytometry. The mean fluorescence emission at individual amphothericin B concentrations is depicted.

agents in blood are well below the MICs for the organisms. In fact, the MICs determined by conventional methods have been suggested to have limited or no relevance to the therapeutic efficacies of the azoles (21). Our data confirm the proposal of Pore (21). The flow cytometry dose-response effect for, for example, ketoconazole occurs at much lower concentrations than the MIC. Since these are concentrations that are therapeutically achievable in blood, the dose-response effect may be the more relevant in vitro test for the prediction of in vivo efficacy. In support of this theory are resistant yeasts like *Candida glabrata* and *Candida krusei*, which did not exhibit dose responses at all and which are causes of infections which are refractory to azole therapy (12, 27).

In summary, the assessment of antifungal efficacy by flow cytometry with FUN-1 can be used to rapidly determine MFCs and MICs. Since the red fluorescence of FUN-1 is impaired by both drug interference with fungal metabolism and membrane damage, the antifungal activities of compounds with different modes of action can be assessed. The assay can be used in drug development efforts to screen candidate compounds and compare them with each other and with commonly used drugs and may be useful in clinical trials of new antifungal compounds. The technique also does not require multiplication of fungi for

TABLE 2. MFCs for nine *C. albicans* strains determined by flow cytometry

C. albicans strain	MFC (mg/liter)			
	Amphotericin B	Flucytosine	Fluconazole	Ketoconazole
4198	0.5	4	>128	64
3977	0.5	8	>128	>128
3953	0.5	2	>128	>128
4184	0.5	1	>128	64
ATCC 26278	0.5	64	8	>128
ATCC 90028	0.5	8	8	8
ATCC 90029	1	>128	4	8
4120	0.5	1	128	64
4141	1	7	>128	128

accurate and reproducible results. In addition, the system can easily be automated (24).

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