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New Fluorescence Assay for the Quantitation of Fungi

T. COLEMAN,[1] JOSEPH V. MADASSERY,[2] GEORGE S. KOBAYASHI,[2,3] MOON H. NAHM,[2] AND J. RUSSELL LITTLE[1]*

Infectious Diseases Division, The Jewish Hospital,[1] and Department of Pathology, Division of Laboratory Medicine,[2] and Department of Medicine,[3] Washington University Medical Center, 216 South Kingshighway, St. Louis, Missouri 63110

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Quantitative determination of fungal mass is easily achieved with a new procedure that detects particle epifluorescence. Fungi are detected after exposure to a fluorescent stain (Fungiqual; CIBA-GEIGY Corp., Summit, N.J.) by using a fluorescence particle concentration analyzer. This report describes a simple fluorescence method for quantitation of either yeast or mycelial forms of fungi. The nature of the staining reaction was studied, and a practical application of this procedure for determination of fungal susceptibility to an antifungal agent is presented.

Quantitation of yeast cells in suspension is usually accomplished by direct microscopy with a hemacytometer or by colony production on solid media. These methods are tedious and provide reliable estimates of cell numbers only with microorganisms in which there is complete separation of daughter cells following cytokinesis. Such methods are not applicable for quantitation of filamentous molds or yeasts which aggregate after cell division or those that form germ tubes.

When fungi are present in tissues, detection and crude quantitation can be achieved by fluorescent staining with agents, such as Cellufluor (Calcofluor) (2, 3) or Blankophor (6), that bind to fungal polysaccharides. However, superior results (6) have been recently reported with diethanol, a chemically related fluorescent stain also known as Uvitex 2B and currently called Fungiqual (CIBA-GEIGY Corp., Summit, N.J.). Fungiqual has been reported to detect most fungi by binding to chitin and certain glucans (12). Our goal in these studies was to develop a quantitative fluorescence assay for fungal cell mass based on detection of Fungiqual bound to fungi cultured in vitro.

Quantitative determination of fluorescent particles has been advanced by development of instrumentation (Fluorescence Concentration Analyzer [FCA; Baxter Healthcare Corp., Round Lake, Ill.]) which quantitatively detects epifluorescence of immobilized particles. Here we report a simple quantitative procedure for determination of fungal yeasts and hyphae which uses the fluorescent fungal stain Fungiqual and the quantitative capabilities of FCA. We also describe some of the chemical characteristics of Fungiqual.

MATERIALS AND METHODS

Chemicals. Fungiqual, the tetrasodium salt of 4,4′-bis (di(2-hydroxyethyl)amino-4-(3-sulfophenylamino)-1,3,5-triazine-6-ylamino)-stilbene-2,2′disulfonic acid (Fig. 1), was kindly provided by D. G. Braun, CIBA-GEIGY Ltd., Basel, Switzerland. The Fungiqual preparation used throughout this study contained about 23% active substance as determined spectrophotometrically by comparison with a Fungiqual standard of known composition. Fungiqual solutions were prepared with sterile 0.15 M saline (normal saline [NS]), and all concentrations noted represent the final concentrations used for staining. Amphotericin B (AmB; E. R. Squibb & Sons, Princeton, N.J.) was reconstituted in distilled water just before use and diluted with glucose-yeast extract medium (2% glucose, 1% yeast extract [GYE]). All other chemicals were reagent grade.

Organisms. *Histoplasma capsulatum* G217B, *Cryptococcus neoformans* capsular strains (A, A145, and A184) and acapsular strains (A32, A59, and A61), and a clinical isolate of *Candida* (*Torulopsis*) *glabrata* were grown at 37°C in GYE. *Mucor* sp. and *Aspergillus niger* were grown at 30°C in GYE, and *Candida albicans* was grown at 25°C in GYE. The culture methods used were as previously described (17). Clinical isolates of *Neisseria gonorrhoeae* and other bacteria were provided by S. Moser, Director of the Microbiology Laboratory, Department of Pathology, Jewish Hospital of St. Louis.

Quantitation of fungi by Fungiqual fluorescence intensity. Fungi and their products were exposed to Fungiqual, the unbound Fungiqual was removed, and the fluorescence of the stained microorganisms was determined by using either an FCA or an SLM 4800 fluorometer (SLM Instruments, Inc., Urbana, Ill.). Fungi were exposed to various concentrations of Fungiqual (0.0000005 to 0.5% [wt/vol]) for 10 to 30 min at room temperature. Following exposure, unbound Fungiqual was removed by filtration or centrifugation and the stained fungi were rinsed twice with NS. Alternatively, fungi were stained, rinsed, and analyzed in FCA plates. FCA assay plates have 96 wells, and each well has a 0.12-ml capacity and a 0.2-μm-pore-size cellulose acetate filter at its bottom. Each FCA plate has a vacuum port which enables particle retention and rinsing on the filter of each well. Epifluorescence from washed particles in each well was detected by using a 400- to 450-nm filter (sample channel). When individual experiments involved collection of data from FCA plates harvested at different times, a uniform amount of fluorescent reference polystyrene particles (Baxter) was mixed with the working stock solution of Fungiqual and used as an internal standard of fluorescence intensities. Fluorescent reference particles were 0.74-μm-diameter polystyrene beads which fluoresce at 590 to 620 nm (reference channel) with essentially no overlap into the 400- to 450-nm sample channel. The optimal dilution of reference particles was 1,000- to 2,000-fold. Quantitative fluorescence in the sample channel divided by fluorescence in the reference channel is referred to as the fluorescence ratio (see Fig. 2).

An SLM 4800 fluorometer was used to evaluate the fluorescence properties of soluble microbial products (*C.*

* Corresponding author.

neoformans capsular polysaccharide, etc.) and some preparations of intact organisms. Unbound Fungiqual was removed from soluble polymers by exhaustive dialysis at 4°C against 10 changes of NS. Emission spectra were collected from 300 to 500 nm at 1-nm intervals with excitation at 345 nm. The sample temperature was maintained at 30°C during these measurements.

Clonogenic assay. The number of *C. glabrata* yeast cells was estimated by production of CFUs. Yeast cells were serially diluted, inoculated, and uniformly spread onto 100-mm-diameter petri plates containing GYE-agar. Macroscopic colonies were counted after overnight incubation at 37°C.

Fungal susceptibility to AmB. *C. glabrata*, *H. capsulatum*, or other fungi were cultured at optimal temperatures (see above) for specified intervals of up to 2 days in 2 ml of GYE medium containing various concentrations of AmB. Samples (0.1 ml) of uniformly suspended yeast cells were transferred to FCA wells, washed twice with NS, exposed for 10 min to 0.075 ml of 0.001% Fungiqual, and rinsed twice with NS, and the fluorescence was determined by FCA. The 50% inhibitory concentration was determined graphically as the concentration of AmB at the midpoint of the difference between the culture inoculum (zero growth) and the control culture after growth without AmB.

RESULTS

Characteristics of the Fungiqual staining system. Fungi and some of their extracellular polysaccharide products were fluorescent after treatment with Fungiqual. Fluorescence, expressed either as fluorescence units or as a fluorescence ratio (with fluorescent reference particles as an internal standard [see Materials and Methods]), was directly proportional to the number of *H. capsulatum* yeast cells (Fig. 2). These data were obtained with samples of suspended viable yeast cells collected in wells of an FCA plate and stained with 0.001% Fungiqual. The concentration of suspended yeast cells was determined in a hemacytometer. In similar experiments, it was found that increased yeast cell fluorescence was produced by increasing the Fungiqual concentration in the staining solution. Fluorescence of *H. capsulatum* stained with Fungiqual was linear with respect to the Fungiqual concentration (0.0005 to 0.05%; data not shown).

Fungiqual solutions were susceptible to chemical degradation and photodegradation. The absorbance maximum of a freshly prepared 0.0245% Fungiqual solution at 349 nm was shifted to 322 nm with a 64% decrease in absorbance at the respective wavelength maximum after exposure to fluorescent light (8 cm from a desktop light source [ITT F15T 8/WW]) for 24 h at room temperature. Fungiqual was also degraded by sodium hypochlorite (0.006%), which decreased the fluorescence of Fungiqual-treated *H. capsulatum* by 86%. However, pathogenic organisms could be killed with 1% formaldehyde, rinsed twice with NS, and exposed to

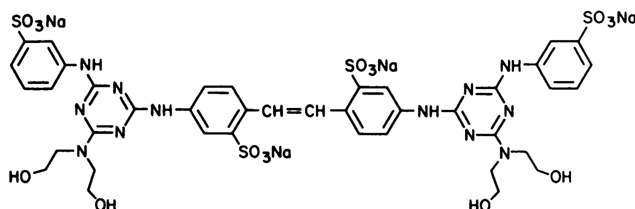

FIG. 1. Molecular structure of Fungiqual.

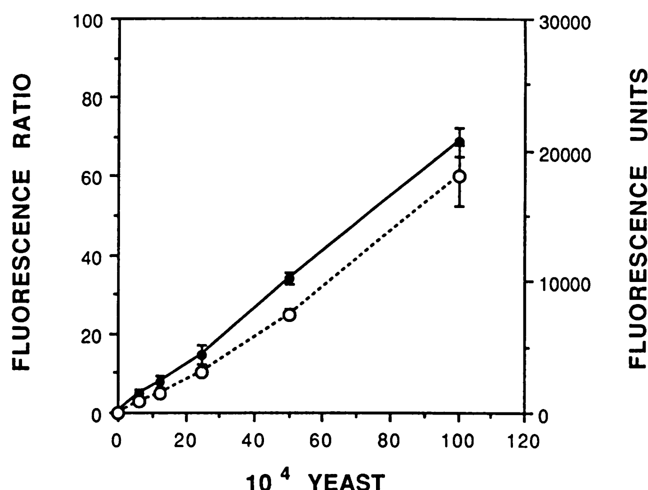

FIG. 2. Fluorescence of Fungiqual-stained *H. capsulatum* yeast cells. Yeast cells were exposed to 0.001% Fungiqual for 10 min at room temperature, rinsed twice with 0.15 M NaCl, and analyzed by FCA. Symbols: ○, fluorescence units; ●, fluorescence ratio.

Fungiqual with no loss of staining efficiency. Formaldehyde-killed *H. capsulatum* exhibited a fluorescence equal to that of viable yeast cells (Table 1).

Fungal staining by Fungiqual was rapid and stable. When equal portions of *H. capsulatum* were exposed to 0.001% Fungiqual for intervals of 2.5 to 40 min and then washed, the fluorescences of different samples measured by FCA were indistinguishable (data not shown).

Relative fluorescences of various fungi. The fluorescence intensity of Fungiqual-treated yeast cells varied with the fungal species. Figure 3 illustrates the fluorescences of three species of fungi stained and washed simultaneously. Twofold dilutions of *C. neoformans*, *C. albicans*, and *H. capsulatum* resulted in fluorescences proportional to the numbers of stained yeast cells. *C. neoformans* gave the greatest overall fluorescence, and *H. capsulatum* gave the least. Most strains of *C. neoformans* (e.g., A, A145, and A184) produce a polysaccharide capsule, while mutant strains (A32, A59, and A61) produce little, if any, capsular polysaccharide (8–10). Direct comparisons of equal numbers of yeast cells showed that strains which produced a capsule displayed greater fluorescence than did acapsular strains (data not shown).

Binding of Fungiqual by chitin and other natural polymers. The finding that capsular strains of *C. neoformans* produced

TABLE 1. Fluorescence of viable and formaldehyde-killed *H. capsulatum*

No. of cells (10^5)	Fluorescence U[a]	
	Viable cells	Formaldehyde-killed cells
100.0	35,740	39,688
50.0	18,415	21,333
25.0	10,935	11,098
12.5	6,723	6,729
6.3	4,660	4,908
3.1	3,310	3,489
1.6	2,845	3,134
0.0	2,050	2,210

[a] Viable or formaldehyde-killed yeast cells were exposed to 0.001% Fungiqual for 10 min, rinsed with 0.15 M NaCl, and analyzed by FCA. Standard deviations were less than 10%.

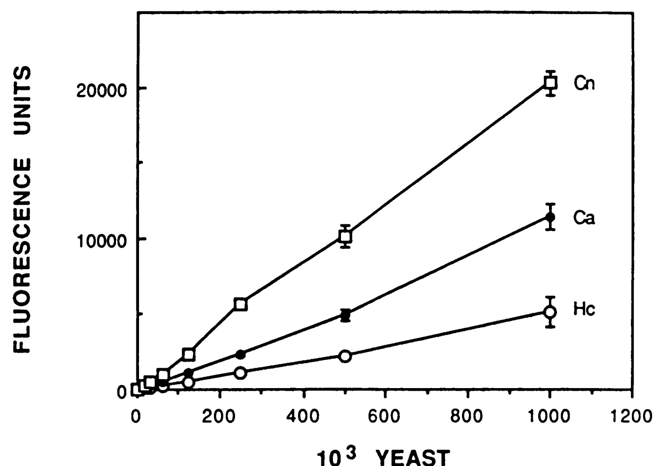

FIG. 3. Fluorescence of three yeast species stained with 0.001% Fungiqual, washed with NS, and assayed by FCA. Abbreviations: Cn, *C. neoformans*; Ca, *C. albicans*; Hc, *H. capsulatum*.

TABLE 2. Use of the Fungiqual emission ratio (E435/E420) to detect binding to microbial particles

Sample	E435/E420 ratio
Fungiqual (0.001%)	1.11
Purified crab chitin (25 μg/ml)	0.89
H. capsulatum (10^7 cells/ml)	0.89
C. albicans (10^7 cells/ml)	0.85
C. neoformans (10^7 cells/ml)	0.85
Capsule from *C. neoformans* (5 mg/ml)	0.89
Saccharomyces cerevisiae mannan (10 mg/ml)	0.97
Fish milt DNA (3.3 mg/ml)	0.95
Dextran T40 (10 mg/ml)	1.06
Dextran sulfate (10 mg/ml)	1.11
Heparin (1,000 U/ml)	1.09
N. gonorrhoeae (A_{730}, 7.9)	1.14
N. meningitidis vaccine (0.4 mg/ml)[a]	1.12

[a] Meningococcal polysaccharide vaccine from serogroups A, C, Y, and W-135.

greater fluorescence than did acapsular strains led to speculation that the capsule of this yeast might bind Fungiqual directly. Purified *C. neoformans* capsular polysaccharides are water-soluble, complex polymers composed mainly of glucuronoxylomannan with a less abundant fraction consisting of galactoxylomannan (14). Polysaccharides were purified from strain A culture supernatant (9); the emission spectra of free and capsular polysaccharide-bound Fungiqual are shown in Fig. 4. The emission wavelength maximum of Fungiqual was shifted from 435 to 420 nm as a consequence of binding to soluble *C. neoformans* capsular material. Essentially identical fluorescence emission spectral shifts were produced when Fungiqual was bound to purified particulate chitin or three different species of intact yeast cells. The ratio of the emission intensity at 435 nm to that at 420 nm (E435/E420 ratio) was chosen to represent differences between free and bound Fungiqual. Free Fungiqual has a E435/E420 ratio of 1.11. Complexes of Fungiqual with

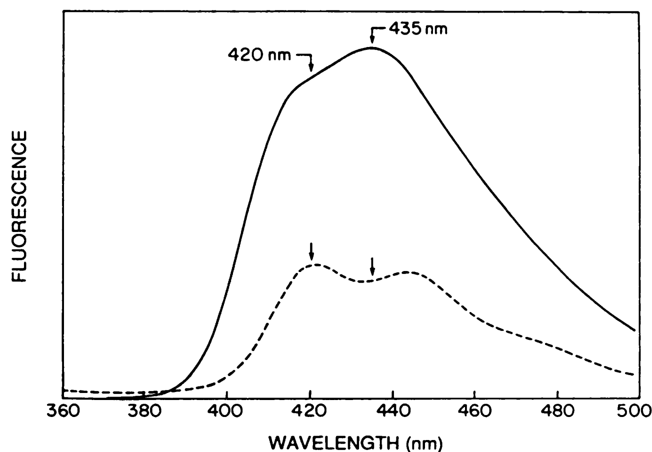

FIG. 4. Fluorescence emission spectrum of 0.001% Fungiqual (solid line) or the capsular polysaccharide from *C. neoformans* treated with 0.001% Fungiqual. The polysaccharide was exposed to Fungiqual for 10 min, exhaustively dialyzed against NS, and analyzed with a SLM 4800 fluorometer (broken line). The wavelengths of interest (420 and 435 nm) are marked by arrows. Excitation was constant at 345 nm.

chitin, yeast, or the capsular polysaccharide from *C. neoformans* had E435/E420 ratios of 0.85 to 0.89 (Table 2). The residual (supernatant) Fungiqual used to stain yeast cells had an emission ratio essentially identical to that of free Fungiqual (data not shown).

The fluorescence emission data in Table 2 are consistent with the idea that binding of Fungiqual by fungi is relatively specific. Dextran T40 bound little, if any, Fungiqual. Dextran sulfate showed slightly greater binding, but the E435/E420 ratio was identical to that of free Fungiqual. Mannan bound very little Fungiqual, but the E435/E420 ratio of the bound Fungiqual decreased slightly to 0.97. Additional polymers that were evaluated for the potential to interact with Fungiqual were heparin and a multiantigenic polysaccharide vaccine from four strains of *N. meningitidis* (Connaught Laboratories, Inc., Swiftwater, Pa.). These carbohydrate polymers bound little Fungiqual, and only minimal shifts in the E435/E420 ratio were detected. Purified DNA fluoresced strongly when exposed to Fungiqual, and its E435/E420 ratio was shifted to 0.95. *N. gonorrhoeae* was fluorescent microscopically after being mixed with Fungiqual, but after washing, spectral analysis indicated that the bacteria did not fluoresce appreciably. Microscopic and FCA evidence indicated that the encapsulated bacteria *Streptococcus pneumoniae* (type 3) and *Haemophilus influenzae* (type b) did not interact with Fungiqual (data not shown).

Susceptibility of yeasts to antifungal agents. The susceptibility of *C. glabrata* to AmB was determined by parallel clonogenic assays and FCA. The 50% inhibitory concentration of AmB for *C. glabrata* was about 0.32 μg/ml by both methods (Fig. 5A and B). The susceptibilities of *H. capsulatum* G217B yeast cells and *Mucor* sp. and *A. niger* hyphae to AmB were also determined by FCA. The 50% inhibitory concentrations of AmB were 0.03 μg/ml for *H. capsulatum* G217B (Fig. 6), 0.14 μg/ml for *Mucor* sp. (Fig. 7), and 0.10 μg/ml for *A. niger* (data not shown).

DISCUSSION

Fungiqual is a fluorescent stilbene derivative that binds a variety of polysaccharides (12). Previous laboratory use of this reagent has been limited to microscopic examinations of fungi and algae (6, 7, 11, 13, 16). We demonstrated a broader

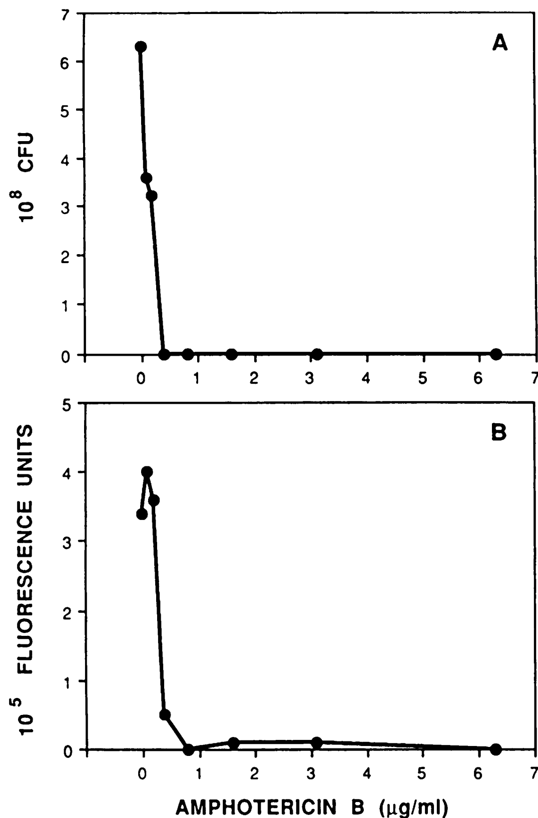

FIG. 5. (A) Susceptibility of *C. glabrata* to AmB as determined by clonogenic assay. *C. glabrata* was exposed to various levels of AmB at room temperature for 6 h. Each sample was serially diluted, and 0.1 ml was placed in a 100-mm-diameter petri dish and mixed with 10 ml of 1% low-melting-temperature agarose maintained at 40°C. The yeast-embedded agarose solidified rapidly and was incubated overnight at room temperature. Colonies which formed after overnight growth were counted. The values plotted are means of colony counts on three different assay plates. Standard deviations averaged 13.4%. (B) Susceptibility of *C. glabrata* to AmB as determined by FCA. Cultures of *C. glabrata* identical to those assayed by the clonogenic method (A) were exposed to AmB. Serial dilutions were prepared as required and analyzed by FCA with 0.001% Fungiqual. Standard deviations averaged 2.8%.

use of Fungiqual by incorporating it into a quantitative assay for fungal mass.

The procedure described in this report provides a simple and sensitive method for detection and quantitation of fungi. Briefly, fungal particles were exposed to Fungiqual, the residual unbound stain was removed, and fluorescent organisms were detected by FCA. Fluorescence intensity was proportional to the number of microorganisms stained (Fig. 2). Binding of Fungiqual was rapid, as maximum staining intensity was reached within 5 min (data not shown). The fluorescence intensity of stained particles was proportional to the Fungiqual concentration (0.000005 to 0.05%) used. Fungiqual concentrations must be chosen judiciously, because the cellulose acetate filters of the FCA analytical plate were stained by Fungiqual. Objectionable levels of background fluorescence by empty wells occurred if the FCA plate filters were exposed to high concentrations of dye (e.g., >0.01%). Optimal sensitivity was obtained by adjusting the Fungiqual concentration empirically (0.001 to 0.01%) to meet the detection requirements while minimizing the background signal. Detection of fungi that have a low affinity for Fungiqual, for example, *C. glabrata* (1; Fig. 5), was accomplished by staining the organism with 0.01% dye, rinsing the stained cells by centrifugation, and suspending the yeast cells before samples of the suspension were loaded into FCA wells. This technique, which provided enhanced sensitivity, allowed detection of about 1,000 *H. capsulatum* yeast cells under optimal conditions. Although Fungiqual was degraded by prolonged exposure to light at room temperature, degradation of Fungiqual was insignificant if solutions were stored at 4°C in light-shielded containers. Organisms killed with formaldehyde were rinsed to remove residual formaldehyde and processed by using routine methods for Fungiqual

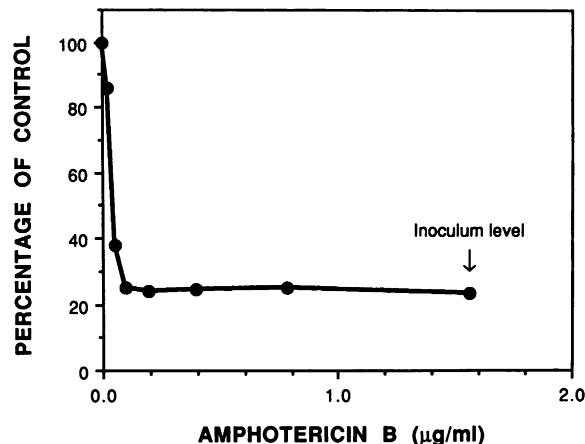

FIG. 6. Susceptibility of *H. capsulatum* yeast cells to AmB. Yeast cells were exposed to various levels of AmB for 2 days at 37°C. Serial dilutions were prepared and analyzed by FCA with 0.001% Fungiqual. The ordinate values were calculated relative to the control culture (100%), which contained no AmB.

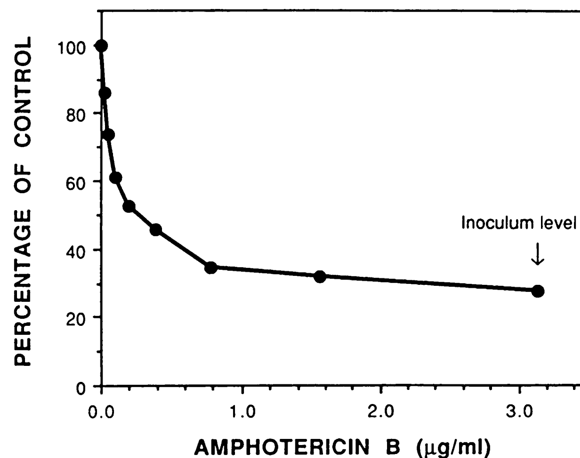

FIG. 7. Susceptibility of *Mucor* sp. to AmB. Hyphal fragments of *Mucor* sp. of a uniform size were prepared by passing a suspended portion of a mycelial mat through a sterile 105-μm polytetrafluoroethylene (TFE) mesh filter (Spectrum Medical Industries, Inc., Los Angeles, Calif.). The turbidity of the hyphal suspension was measured at 730 nm, different amounts of AmB were added, and the turbidity was adjusted to an arbitrary initial level of 0.1. Hyphae exposed to AmB were incubated for 6 h at 30°C, and samples were diluted as required, exposed to 0.001% Fungiqual, and assayed by FCA. Ordinate values were calculated as described in the legend to Fig. 6.

staining and fluorescence detection (Table 1). Analysis of pathogenic microorganisms was safe and easy when this procedure was used.

Equal numbers of yeast cells from three different species exhibited different fluorescence intensities when stained with the same concentration of Fungiqual (Fig. 3). The reasons for differences in fluorescence among different species are unclear, but several factors may contribute to variations in particle staining. For example, cell size and wall composition probably influence the extent and manner of Fungiqual binding.

The fluorescence emission spectrum of free Fungiqual exhibited a unimodal shape with a wavelength maximum at 435 nm and a shoulder near 412 nm. When Fungiqual bound to the soluble capsular polysaccharide of *C. neoformans* (predominately glucuronoxylomannans), the shape of the emission spectrum was bimodal, with peaks at 420 and 443 nm (Fig. 4). Similar spectral shifts (quantitated by E435/E420 ratios) were observed when Fungiqual was bound to intact *H. capsulatum*, *C. albicans*, or *C. neoformans* (Table 2). In contrast, capsular polysaccharides from type 3 pneumococci, meningococci, or *H. influenzae*, as well as unsubstituted mannan or dextran, had little or no interaction with Fungiqual as determined by lack of visible fluorescence and/or insignificant alterations of the E435/E420 ratio of Fungiqual-treated preparations (Table 2). Type 3 *S. pneumoniae* has a capsule with repeating units of glucose and glucuronic acid, while the capsule of *H. influenzae* type b is a linear polymer of ribosyl-ribitol phosphate.

It seems likely that Fungiqual binds to polymers with its four hydroxyl groups (12; Fig. 1), but it may also intercalate between or within long polymeric coils of the fungal wall matrix. Regardless of the mechanism of binding, the emission spectral shift of bound Fungiqual suggests that binding may change either the conformation or the molecular environment of this fluorescent molecule.

Susceptibility of fungal pathogens to antifungal agents is traditionally assayed in vitro by determination of MICs, usually by analysis of CFUs or turbidity. This procedure is laborious and subject to error with organisms such as *H. capsulatum* that tend to aggregate in suspension (4, 5). To determine the feasibility of the FCA method for susceptibility testing, parallel assays using CFU and FCA detection were performed with *C. glabrata*. Previous reports indicated that this yeast shows minimal aggregation in suspension and does not form germ tubes. The 50% inhibitory concentration of AmB for *C. glabrata* was estimated to be 0.32 μg/ml by both methods (Fig. 5A and B), which is in agreement with a previous report (15). When the same method was used to quantitate the AmB susceptibility of *H. capsulatum* G217B, a *Mucor* sp., and *A. niger*, the 50% inhibitory concentrations were, respectively, 0.03 μg/ml (Fig. 6), 0.14 μg/ml (Fig. 7), and 0.10 μg/ml (data not shown).

This report describes a new quantitative fluorescence assay for fungi. The assay procedure uses the fluorescent fungal stain Fungiqual and is quantitated by use of FCA. This method is rapid and sensitive and can be adapted to assay pathogenic fungi under a variety of conditions. Because of its relatively specific staining of fungi, it can be used with complex mixtures involving fungal pathogens and host cells such as macrophages (data not shown). The technique has potential applications to a variety of studies directed at quantitation of the enhancement or inhibition of fungal growth in vitro.

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ADDENDUM IN PROOF

Two recent articles have appeared on the staining of fungi by Fungiqual (Uvitex 2B) in histological tissue sections (E. D. Wachsmuth, Histochem. J. **20**:215–221, 1988; E. D. Wachsmuth, Virchows Arch. B Cell Pathol. **56**:1–4, 1988).

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