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Sensitivity of *Candida albicans* Germ Tubes and Biofilms to Photofrin-Mediated Phototoxicity

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Treatment of mucocutaneous and cutaneous *Candida albicans* infections with photosensitizing agents and light, termed photodynamic therapy (PDT), offers an alternative to conventional treatments. Initial studies using the clinically approved photosensitizer Photofrin demonstrated the susceptibility of *C. albicans* to its photodynamic effects. In the present study, we have further refined parameters for Photofrin-mediated photodynamic action against *C. albicans* and examined whether mechanisms commonly used by microorganisms to subvert either antimicrobial oxidative defenses or antimicrobial therapy, including biofilm formation, were operative. In buffer and defined medium, germ tubes preloaded with Photofrin retained their photosensitivity for up to 2 hours, indicating the absence of degradation or export of Photofrin by the organism. The addition of serum resulted in a gradual loss of photosensitivity over 2 hours. In contrast to an adaptive response by germ tubes to oxidative stress by hydrogen peroxide, there was no adaptive response to singlet oxygen-mediated stress by photodynamic action. *C. albicans* biofilms were sensitive to Photofrin-mediated phototoxicity in a dose-dependent manner. Finally, the metabolic activity of *C. albicans* biofilms following photodynamic insult was significantly lower than that of biofilms treated with amphotericin B for the same time period. These results demonstrate that several of the mechanisms microorganisms use to subvert either antimicrobial oxidative defenses or antimicrobial therapy are apparently not operative during Photofrin-mediated photodynamic treatment of *C. albicans*. These observations provide support and rationale for the continued investigation of PDT as an adjunctive, or possibly alternative, mode of therapy against cutaneous and mucocutaneous candidiasis.

The fungus *Candida albicans* commonly colonizes the epithelial surfaces of the body, with the oropharyngeal cavity and the vaginal tract as primary sites of mucosal colonization (4). Impairment of innate and adaptive host defenses, perturbation of normal bacterial flora, or underlying disease can contribute to fungal overgrowth and penetration of these mucosae by *C. albicans* (5, 36). Chronic exposure to excessive moisture is a predisposing factor for cutaneous candidiasis, and keratinized surfaces are also susceptible to infection (4). *C. albicans* grows as a biofilm on epithelial surfaces (10, 20) and prosthetic devices (23, 34), contributing to the failure of antifungal therapy and recurrent infection.

Treatment of superficial *C. albicans* infections with photosensitizing agents and light, termed photodynamic therapy (PDT), offers an alternative to conventional treatments (8, 9). Derived from the acid treatment of hematoporphyrin, the clinically approved photosensitizer Photofrin is used in the United States for the treatment of endobronchial and esophageal tumors (27) and was recently approved for the treatment of Barrett's esophagus (21). In the presence of molecular oxygen, the irradiation of photosensitizers such as Photofrin with the appropriate wavelength of light results in the local production of singlet oxygen, which rapidly oxidizes cellular macromolecules found nearby, leading to cell damage and death (9). Initial studies (3) using Photofrin demonstrated the susceptibility of *C. albicans* to its photodynamic effects. In the present study, we have further refined parameters for Photofrin-mediated photodynamic action against *C. albicans* and examined whether mechanisms commonly used by microorganisms to subvert either antimicrobial oxidative defenses or antimicrobial therapy, including biofilm formation, were operative.

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MATERIALS AND METHODS

***C. albicans* germ tube growth conditions.** *C. albicans* laboratory strain 3153A (31) was grown overnight at 37°C on a shaker platform in liquid yeast extract-peptone-dextrose (YEPD) medium (Difco, Detroit, MI) with vigorous aeration (225 rpm) to stationary phase (~2×10^8 to 3×10^8 cells/ml). Under these conditions, organisms were >99% blastoconidia. One ml of culture was washed twice with distilled H_2O and diluted to 3×10^5 cells/ml in RPMI 1640 supplemented with 1% glucose (BioWhittaker, Walkersville, MD). To induce filament formation, 3 ml of the blastoconidium suspension was grown statically in six-well tissue culture dishes (VWR) at 37°C for 3 h. For microscopy, a sterile 22-mm-square glass coverslip of thickness no. 1 was placed in the well as a substrate for organism adherence. The germination frequency was >95%. Although some of the organisms had septated filaments indicative of cell division, for clarity these forms will be referred to as germ tubes in order to distinguish them from the hyphal forms in the *C. albicans* biofilms described below. Prior to incubation with Photofrin, germ tubes were washed with Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium (DPBS) (Invitrogen, Carlsbad, CA) containing 0.1% glucose (DPBSG) to remove nonadherent cells.

***C. albicans* biofilm formation.** To provide a substrate for biofilm formation, 2 ml of fetal bovine serum (6) was added to each well of a six-well tissue culture dish and incubated at 37°C overnight with gentle rocking. *C. albicans* strain 3153A was grown overnight in YEPD and washed twice with distilled H_2O as described previously for germ tube formation. Serum-coated tissue culture wells

were washed twice with PBS to remove excess serum. Preliminary studies demonstrated that, of several media tested, RPMI 1640 plus 1% glucose resulted in the most robust biofilm growth. Washed *C. albicans* cells (3×10^5 cells/ml) in 3 ml RPMI 1640 plus 1% glucose were added to each well and incubated at 37°C for 90 min with gentle rocking to initiate attachment and biofilm formation. Nonadherent cells were removed after gentle washing with DPBSG, and 3 ml of fresh RPMI 1640 plus 1% glucose was added to each well. Cultures were incubated at 37°C with gentle rocking for up to 48 h for biofilm formation.

Biofilms formed after 6 to 8 h were tightly adherent. After 24 h of incubation, the tightly adherent layer was accompanied by a loosely associated upper layer, which could be dispersed, at least partly, by vigorous washing. After 48 h, this loose upper layer became too fragile to permit manipulation. Hence, after the initial study using biofilms grown up to 48 h, 24-h biofilms were utilized.

Phototoxicity assay of germ tubes and biofilms treated with Photofrin. For photodynamic treatment, *C. albicans* germ tubes or biofilms were incubated with Photofrin (Axcan Pharma, Birmingham, AL) in DPBSG at 37°C for the time periods and concentrations indicated for each experiment. In most cases, cells were washed twice with DPBSG after incubation with Photofrin to remove excess photosensitizer, and 2 ml fresh DPBSG was added for the irradiation step. However, due to the fragile nature of the upper layer of the biofilm, Photofrin was not washed away prior to irradiation for experiments examining the effect of total fluence on Photofrin-mediated phototoxicity against biofilms (see Fig. 6) or for the comparison of Photofrin-mediated phototoxicity with toxicity due to amphotericin B. In one experiment (see Fig. 5), cells preloaded with Photofrin were incubated in different media as indicated prior to irradiation.

For irradiation, the cover of the six-well dish was removed and the plates were irradiated with broadband visible light from an Hg arc lamp (Olympus BH2-RFL-T2; Olympus Optical Co., Ltd., Tokyo, Japan) reflected to the sample with a cold mirror (Edmund Industrial Optics, Barrington, NJ), whose reflection and transmission properties were such that the spectrum of the beam at the sample was limited to 400 to 700 nm. Irradiation was performed at room temperature at a fluence rate of 15 mW/cm^2 as measured by a power/energy meter (model 13PEM001; Melles Griot, Carlsbad, CA). This fluence rate resulted in a fluence of 0.9 J/cm^2 for each minute of irradiation. For each experiment, an identical plate that remained in the dark (nonirradiated) was included as a control.

Following irradiation, organisms were incubated in fresh RPMI 1640 plus 1% glucose for 30 min at 37°C to allow the effect of PDT to manifest itself. The 30-min incubation time allowed for recovery is considerably shorter than the overnight incubation commonly used to evaluate the effects of the photodynamic treatment of tumor cells, due to the comparatively more rapid replication time of the organism. Photofrin-mediated phototoxicity to cells was measured by a (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide (XTT; Sigma, St. Louis, MO) assay that utilizes a previously described method (29) in which XTT is metabolized by mitochondrial dehydrogenases to a soluble, orange-colored formazan product that diffuses into the surrounding medium. Briefly, XTT was freshly prepared in DPBS (without calcium, magnesium, or added glucose), heated at 60°C for 30 min, and filtered. Coenzyme Q (Sigma) was mixed with the XTT, and the solution was added to cells at final concentrations of 0.5 mg/ml XTT and 40 μg/ml coenzyme Q. Plates were incubated at 37°C for 1 h, at which time 100 μl of the reaction supernatant was removed and diluted with an equal volume of PBS in a microtiter well. The intensity of the colorimetric reaction, reflecting cell metabolic activity, was calculated by measuring the optical density at 450 nm (OD_{450}) using an automated plate reader (Bio-Rad Laboratories, Hercules, CA). A reduction in the OD_{450} in irradiated cultures compared to that in nonirradiated cultures was taken as a measure of organism damage (3, 29).

Fluorescence microscopy. Adherent *C. albicans* germ tubes were prepared on 22-mm-square glass coverslips of thickness no. 1 as described above. One set of samples was incubated with 5 μg/ml of Photofrin in DPBSG for 5 min, washed with DPBSG, rinsed briefly with water, dried, and examined using fluorescence microscopy. A second set of samples was treated identically and subjected to irradiation. Irradiated and nonirradiated samples were incubated with 10 μg/ml YO-PRO-1 in PBS for 10 min, washed, dried, and examined using fluorescence microscopy. Fluorescence microscopy was performed using a trinocular Olympus BX41 microscope prefitted with a fluorescein filter cube for the detection of YO-PRO-1 uptake. The uptake of Photofrin was visualized by fluorescence microscopy using a custom porphyrin filter set and filter cube (Ex. 405/30, Dichroic 440 LP, Em. OG 590; Chroma Technology Corp., Rockingham, VT). Fluorescence photomicrographs of YO-PRO-1 uptake in irradiated and nonirradiated samples were taken under the same conditions using identical exposure times of 0.5 s. The electronic manipulations of these fluorescence photomicrographs in PhotoShop (Adobe Systems, Inc., San Jose, CA) were also identical.

Adaptive response to oxidative stress. Pilot studies were performed to confirm that *C. albicans* strain 3153A demonstrated an adaptive response to oxidative stress induced by H_2O_2. To determine the toxicity of H_2O_2, germ tubes were treated with a range of concentrations of H_2O_2 (0 to 100 mM) in RPMI 1640 plus 1% glucose for 1 h. Damage to cells was evaluated by the XTT assay. We confirmed that H_2O_2 concentrations higher than 25 mM were highly toxic, while cells treated with 1 mM H_2O_2 metabolized XTT at a level comparable to that seen for untreated cells. For the adaptive response to H_2O_2, germ tubes were treated first with 1 mM H_2O_2 and then with a second dose of H_2O_2 in a range from 50 to 100 mM. For photodynamic studies, germ tubes were treated with subtoxic concentrations (0.5 μg/ml) of Photofrin for 1 min, irradiated at a fluence of 9 J/cm^2, allowed to recover in fresh RPMI 1640 plus 1% glucose for 30 min, and washed with DPBSG. Germ tubes were then treated with increasing concentrations of Photofrin ranging from 1 to 10 μg/ml for 1 min, a procedure followed by a second round of irradiation at the same fluence and a 30-min recovery period. Metabolic activity was measured using the XTT assay after the completion of either H_2O_2 treatment or PDT.

Subsequent experiments were performed to determine whether primary treatment with a subtoxic concentration of either Photofrin or H_2O_2 increased resistance to the other agent. Primary treatment with 1 mM H_2O_2 was followed by secondary treatment with Photofrin concentrations ranging from 1 μg/ml to 10 μg/ml. Primary treatment with 0.5 μg/ml of Photofrin was followed by secondary treatment with 50 mM H_2O_2. Treatment protocols similar to those described above were used, except that volumes of reagents were scaled down in order to perform the assays in 96-well dishes to more readily accommodate the different treatment combinations.

Comparison of Photofrin-mediated photodynamic action and amphotericin B activity against *C. albicans* biofilms. *C. albicans* was grown in RPMI 1640 supplemented with 1% glucose for 24 h and then treated with 10 μg/ml Photofrin for 30 min. One culture was irradiated at a fluence of 18 J/cm^2, while a parallel culture was not irradiated. Irradiation was followed by a 30-min incubation period in the dark. In parallel, a 24-h biofilm was treated with 10 μg/ml amphotericin B for the duration of photosensitization, irradiation, and handling, approximately 90 min (30-min incubation of parallel biofilms with Photofrin, 20-min irradiation, 30-min recovery period, and 10-min total handling time). This concentration of amphotericin B is severalfold higher than the serum levels (0.5 to 2.0 μg/ml) used in the treatment of patients with *C. albicans* infection (33) and was designed to expose the organisms to potentially toxic levels of drug. Amphotericin B-treated biofilms were incubated in the dark. Excess Photofrin or amphotericin B was removed by washing with DPBSG immediately prior to the XTT assay. The metabolic activities of all the biofilms were measured by using the XTT assay as described for germ tubes.

Statistical analysis. Each experimental group was assayed in duplicate. All experiments were performed three times, with the exception of the experiment describing the effect of photodynamic action on 0- to 48-h biofilms (see Fig. 5), which was performed twice. Data are presented as the mean from combined replicate experiments ± standard deviation. Comparisons between photosensitized toxicities for irradiated and nonirradiated samples in the same group and between-group comparisons were made using the Student *t* test. In all cases, *P* values of ≤ 0.05 were considered significant.

RESULTS

Optimizing conditions for Photofrin-mediated phototoxicity to *C. albicans* germ tubes. *C. albicans* germ tube monolayers were incubated with Photofrin for different times over a range of concentrations to set parameters for effective photosensitization. Following irradiation and a brief recovery period, the efficacy of treatment was evaluated by measuring the metabolic activity of the organisms with the XTT assay. In all experiments, the metabolic activity of irradiated cells was compared to that of cells treated in an identical manner but not irradiated. A decreased level of XTT metabolism compared to that in control cultures was used as an indicator of organism damage following irradiation (3, 29).

Our initial studies (3) used a 30-min incubation time with Photofrin to load cells, followed by irradiation for 10 min at a fluence rate of 15 mW/cm^2, resulting in a fluence of 9 J/cm^2. Subjecting germ tubes to irradiation using the same fluence of

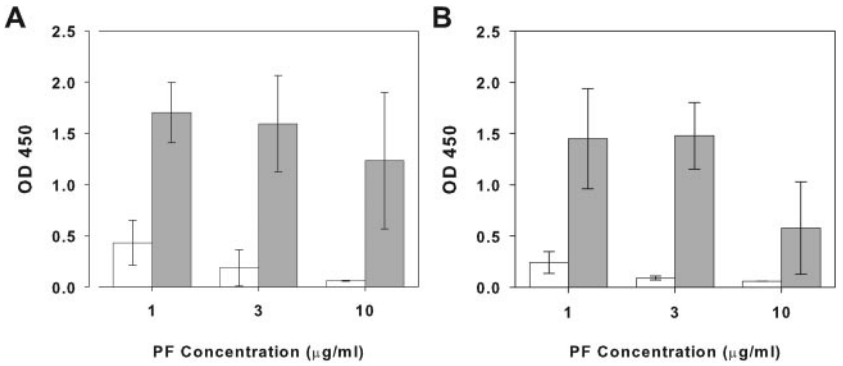

FIG. 1. Dose response of Photofrin phototoxicity against *C. albicans* germ tubes. *C. albicans* germ tubes were incubated with 10 μg/ml Photofrin at 37°C for either 1 or 5 min. One group was irradiated with a fluence of 9 J/cm^2, while the other was not irradiated. The metabolic activities of the germ tubes were measured using the XTT assay, and substrate conversion was determined spectrophotometrically by measuring OD_{450}. Similarly, *C. albicans* germ tubes were incubated with 1, 3, or 10 μg/ml Photofrin for either 1 min (A) or 5 min (B), and metabolic activities were measured by the XTT assay. PF, Photofrin. Open bars, irradiated samples. Closed bars, nonirradiated samples.

9 J/cm^2 following incubation with 10 μg/ml Photofrin for as briefly as 1 to 5 min resulted in the virtually complete elimination of *C. albicans* dehydrogenase enzyme activity as measured by the XTT assay relative to results for nonirradiated cultures. Germ tubes loaded for either 1 (Fig. 1A) or 5 (Fig. 1B) min with a range of concentrations of Photofrin demonstrated significant levels ($P < 0.05$) of organism damage with concentrations as low as 1 μg/ml of Photofrin. The modest reduction in XTT metabolism in nonirradiated organisms incubated with 10 μg/ml compared to that with a lower concentration may be due to a concentration-dependent dark effect or to light-induced effects that result from the unavoidable exposure of Photofrin-only control samples to minimal light during manipulation in the laboratory. Previous studies demonstrated that *C. albicans* germ tubes that were not exposed to Photofrin but were irradiated did not differ significantly, in terms of metabolic activity, from organisms treated with Photofrin but not irradiated (3).

Photofrin-mediated phototoxicity is correlated with lethality to *C. albicans* germ tubes. Since the XTT metabolic assay is a measure of enzyme activity and not cell death, we sought additional criteria to determine whether there was uniform killing of *C. albicans* germ tubes following photodynamic insult with Photofrin. The fluorescent dye YO-PRO-1 (Molecular Probes, Eugene, OR) is excluded by intact plasma membranes; hence, entry of YO-PRO-1 into the cell is indicative of a loss of membrane integrity and death. Phototoxicity against *C. albicans* germ tubes using Photofrin was followed by incubation with YO-PRO-1 and examination of organisms by fluorescence microscopy. Representative examples are shown in Fig. 2. The fluorescence photomicrograph in Fig. 2A and the corresponding bright-field photomicrograph in Fig. 2B demonstrate uniform rates of Photofrin uptake (5 μg/ml) by the organisms after 5 min. Similarly treated organisms irradiated at a fluence of 9 J/cm^2 were uniformly and strongly labeled by YO-PRO-1, as determined by fluorescence (Fig. 2C), which is indicative of a loss of membrane integrity and cell death. The corresponding bright-field photomicrograph is shown in Fig. 2D. In contrast, organisms that were treated with Photofrin but not irradiated demonstrated no fluorescence following incubation with YO-PRO-1 (Fig. 2E; corresponding bright field, Fig. 2F).

Taken together, the data in Fig. 1 and 2 suggest that the actual amount of organism killing may be more extensive than is indicated by the XTT assay, particularly when photodynamic treatment results in baseline levels of XTT metabolism. A CFU-based assay could reveal the true extent of killing but would need to be interpreted carefully, given the tendency of the germ tubes to both adhere to the culture dish and agglutinate in suspension.

Effect of medium on Photofrin retention by *C. albicans* germ tubes over time. To evaluate the retention of Photofrin over time, *C. albicans* germ tubes were incubated with 10 μg/ml Photofrin for 10 min and washed with DPBSG. One group of organisms was irradiated immediately. Other groups were incubated under different conditions for up to 2 h prior to irradiation to determine whether sensitivity to irradiation diminished over time due to either Photofrin efflux or metabolic processing of Photofrin by *C. albicans*. Incubation of Photofrin-loaded organisms in either DPBSG (Fig. 3A) or RPMI 1640 plus 1% glucose (Fig. 3B) for up to 2 h did not diminish the sensitivity of *C. albicans* germ tubes to irradiation.

Previous studies of Photofrin sensitization of tumor cells demonstrated that the addition of serum to cell culture media enhanced the leaching of Photofrin from cells (1, 24). For germ tubes preloaded with Photofrin, the addition of 20% fetal calf serum (FCS) to RPMI 1640 plus 1% glucose resulted in a gradual increase in resistance to irradiation over time (Fig. 3C). After 1 h of incubation in medium containing FCS, preloaded organisms were still significantly more sensitive to irradiation than nonirradiated organisms were, but the sensitivity had diminished considerably compared to organisms irradiated immediately after loading with Photofrin. After 2 h of incubation in the presence of serum, no phototoxicity was observed.

***C. albicans* germ tubes do not display adaptive response to oxidative stress caused by Photofrin-mediated photodynamic insult.** Preliminary experiments established a dose response to H_2O_2, with 1 mM H_2O_2 showing minimal toxicity and doses in excess of 25 mM being significantly toxic (data not shown). As predicted by several earlier reports (15, 19, 33), primary treatment of *C. albicans* germ tubes with 1 mM H_2O_2 induced resistance to a subsequent H_2O_2 challenge. Exposure to a subsequent challenge of 50 mM H_2O_2 resulted in only a mod-

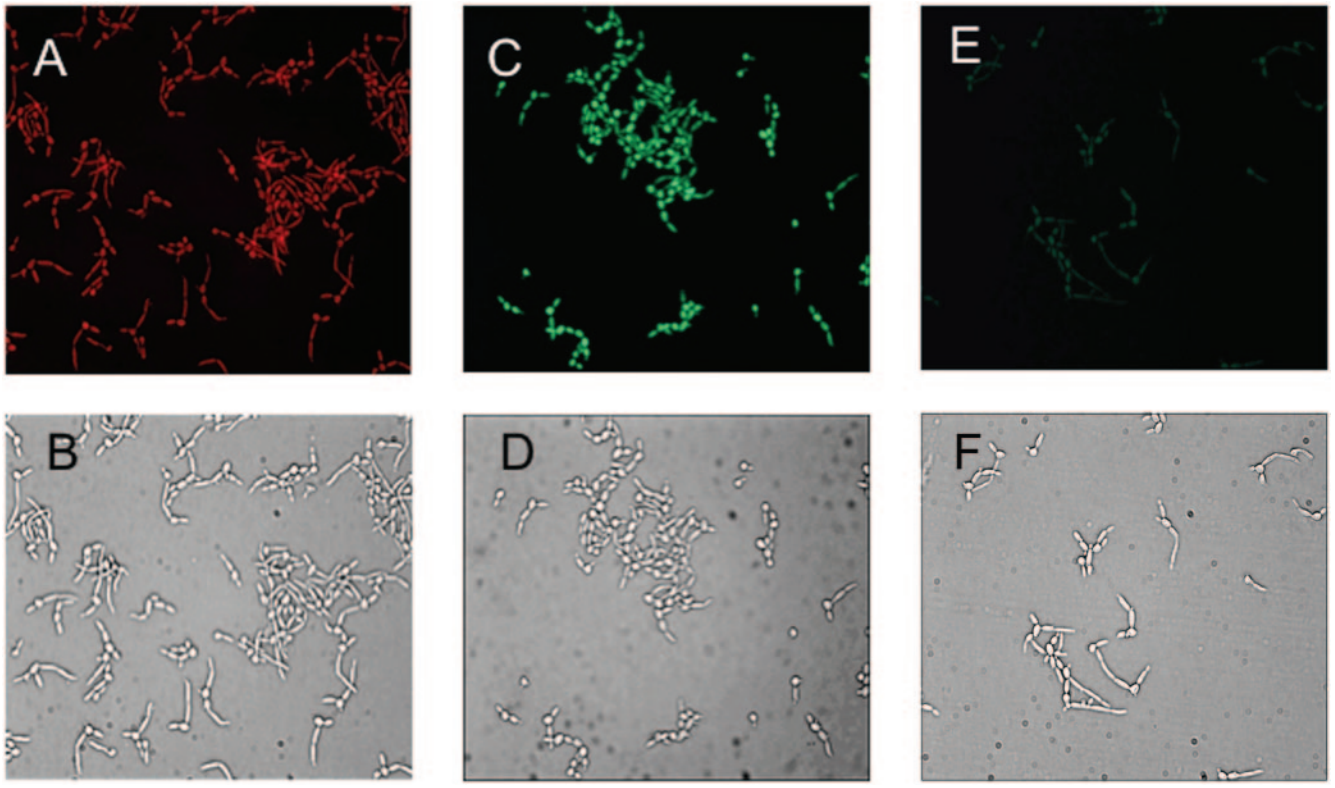

FIG. 2. Photomicrographs of *C. albicans* germ tubes following Photofrin photosensitization. Fluorescence indicating Photofrin (5 μg/ml) uptake after 5 min (A) and corresponding bright field (B). Fluorescence indicating entry of YO-PRO-1 dye into Photofrin-treated, irradiated germ tubes (C) and corresponding bright field (D). Organisms were incubated with 5 μg/ml for 5 min and washed prior to irradiation with a fluence of 9 J/cm^2. Absence of fluorescence in Photofrin-treated but nonirradiated germ tubes indicating no detectable entry of YO-PRO-1 dye (E) and corresponding bright field (F).

est reduction in XTT activity compared to controls, and a subsequent challenge of 100 mM H_2O_2 resulted in an approximately 40% reduction in XTT activity (data not shown). In contrast, primary treatment with a subtoxic concentration of 0.5 μg/ml of Photofrin did not result in increased resistance to a secondary Photofrin treatment with concentrations ranging from 1 μg/ml to 10 μg/ml (Fig. 4A). The phototoxicity to irradiated organisms exposed to these secondary treatments of Photofrin was comparable to that seen using the same Photofrin concentrations as in a primary treatment (Fig. 1).

Subsequent experiments were performed to determine whether there was any cross talk between the organism's responses to these two types of oxidative stress. Primary treatment of *C. albicans* with a subtoxic concentration of 1 mM H_2O_2 and secondary treatment with increasing concentrations of Photofrin ranging from 1 μg/ml to 10 μg/ml did not result in an enhanced resistance to the photosensitizer compared to the resistance of organisms treated with Photofrin alone (Fig. 4B). Similarly, primary treatment of organisms with a subtoxic concentration of 0.5 μg/ml Photofrin and irradiation did not result in enhanced resistance to a secondary treatment with 50 mM H_2O_2 compared to the resistance of organisms treated only with 50 mM H_2O_2 (data not shown).

Photofrin-mediated phototoxicity against *C. albicans* biofilms. Compared to planktonic growth, the sensitivities of both bacteria (14, 17) and fungi (6, 25) to many conventional antimicrobial agents diminish considerably when organisms are grown in biofilms. Therefore, the sensitivity of *C. albicans* biofilms to Photofrin-mediated phototoxicity was examined in organisms cultured over 48 h of biofilm formation to determine whether the same phenomenon occurred.

C. albicans blastoconidia were inoculated in six-well tissue culture dishes at a density of 3×10^5 cells/ml in 3 ml RPMI 1640 tissue culture medium supplemented with 1% glucose and incubated at 37°C for 90 min with gentle rocking. Nonadherent organisms were removed by washing with DPBSG. There are several stages of *C. albicans* biofilm growth. *C. albicans* biofilms that form for at least 6 h are termed early biofilms, those that form for over 20 h are termed intermediate, and those that form for longer than 40 h are termed mature (18). The time course of incubation resulted in the formation of germ tube monolayers from the earliest time point (3 h) to mature biofilm formation at 48 h (18). At intervals, the organisms were tested for photosensitivity with Photofrin. Organisms were treated with 10 μg/ml Photofrin for 30 min at 37°C, excess Photofrin was removed by gentle washing with DPBSG, and biofilms were irradiated at a fluence of 18 J/cm^2. After 30 min of incubation in fresh medium, the metabolic activity of the cells was determined using the XTT assay. As with the germ tube monolayers, control biofilm cultures were treated identically, except that they were not irradiated.

Photofrin-mediated photoxicity against the biofilms was ex-

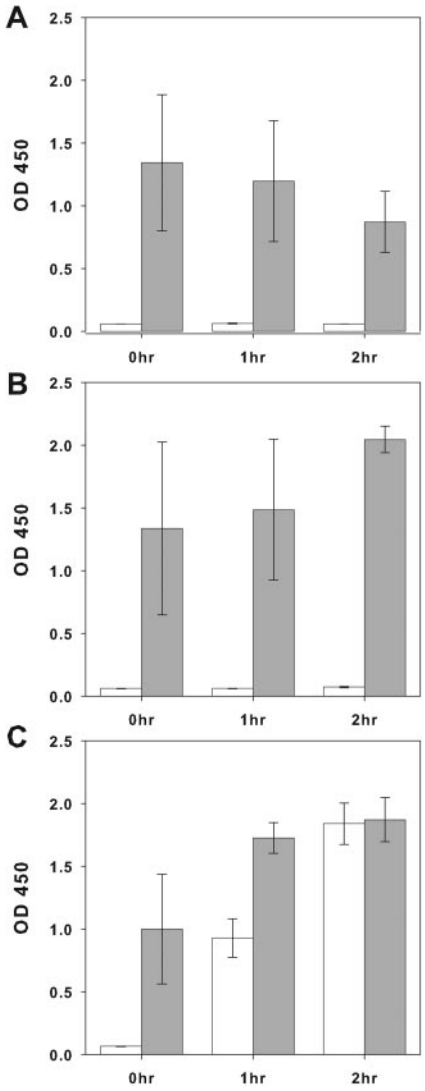

FIG. 3. Effect of medium on Photofrin retention by *C. albicans* germ tubes over time. *C. albicans* germ tubes were loaded with 10 μg/ml Photofrin for 10 min. Prior to irradiation, organisms were incubated in either DPBSG (A), RPMI 1640 supplemented with 1% glucose (B), or RPMI 1640 supplemented with 1% glucose and 20% FCS (C) for 0, 1, or 2 h prior to irradiation. Cultures were irradiated with a fluence of 9 J/cm^2, while a parallel culture was not irradiated. The metabolic activities of the germ tubes were measured using the XTT assay, and substrate conversion was determined spectrophotometrically at 450 nm. Open bars, irradiated samples. Closed bars, nonirradiated samples.

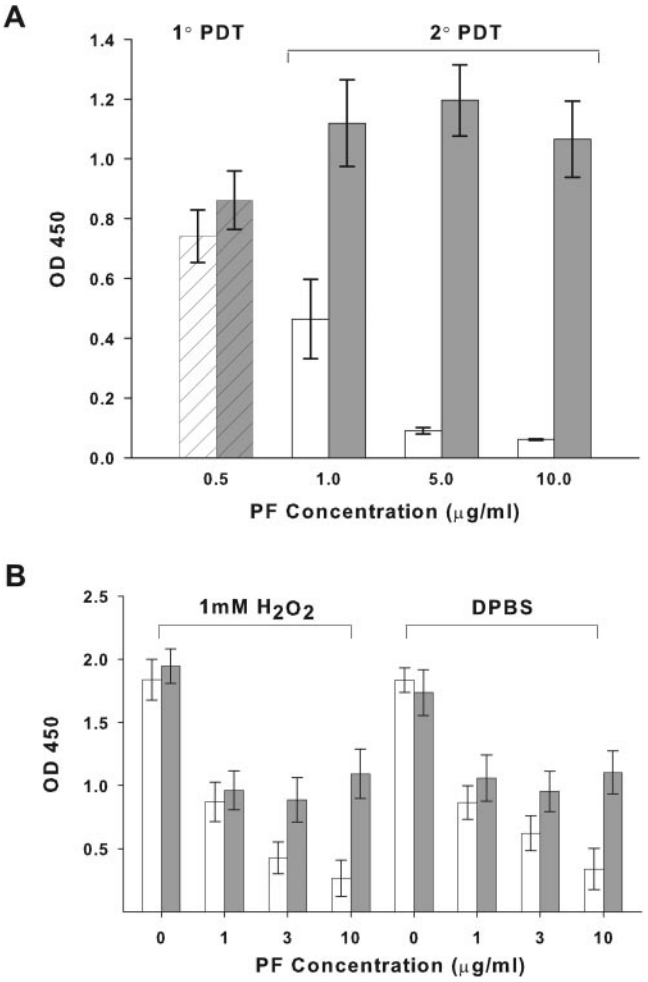

FIG. 4. Lack of an adaptive response in *C. albicans* germ tubes to Photofrin-mediated oxidative stress. (A) *C. albicans* germ tubes were given a subtoxic primary (1° PDT) treatment of 0.5 μg/ml Photofrin for 1 min followed by irradiation at a fluence rate of 15 mW/cm^2 for 10 min, resulting in a fluence of 9 J/cm^2 (bars on far left of graph). After 30 min of incubation in fresh RPMI 1640 plus 1% glucose, germ tubes were incubated with 1, 5, or 10 μg/ml Photofrin for 5 min, and a secondary (2° PDT) irradiation was administered at the same fluence. Control cultures were treated similarly but were not irradiated. (B) *C. albicans* germ tubes were given a primary treatment with either 1 mM H_2O_2 in buffer or buffer alone (DPBS) as indicated, followed by a secondary treatment with increasing concentrations of Photofrin and irradiated as described for panel A. Control cultures were treated similarly but were not irradiated. PF, Photofrin. Open bars, irradiated samples. Closed bars, nonirradiated samples.

tensive and did not diminish over the time course tested (Fig. 5). The reduced metabolic activity in the nonirradiated biofilms at 48 h compared to that at 24 h may be due to cells approaching a more metabolically quiescent state, to cell losses during processing, or both. The treatment with Photofrin and the XTT assay necessitated a number of wash steps, and, in the six-well dishes, the biofilms became more fragile over time. Nonetheless, significant reductions of metabolic activity were observed in irradiated cultures compared to nonirradiated cultures at all time points (P values: 3 h = 0.007; 6 h, 24 h, and 48 h = < 0.001).

Similar to the observations made using *C. albicans* germ tubes (Fig. 1B and C), Photofrin-mediated phototoxicity against *C. albicans* biofilms was concentration dependent, with 1 μg/ml of Photofrin resulting in an approximately 30% reduction of metabolic activity in 24-h biofilms compared to that in nonirradiated controls and higher concentrations of Photofrin leading to proportionally greater organism damage (data not shown).

Effect of fluence on Photofrin-mediated phototoxicity against *C. albicans* biofilms. In addition to determining the lower limits of time of exposure to Photofrin and Photofrin

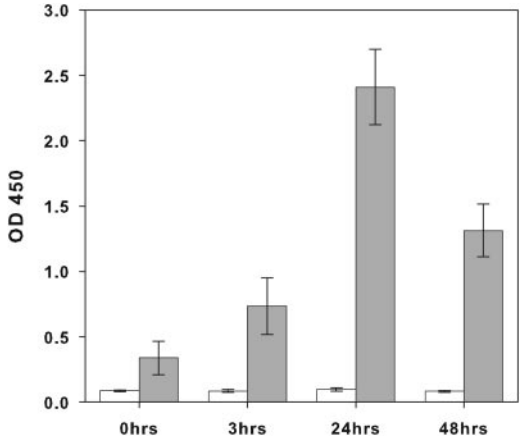

FIG. 5. Photofrin-mediated phototoxicity against *C. albicans* during biofilm maturation. *C. albicans* cells were grown in RPMI 1640 supplemented with 1% glucose for different time periods ranging from 3 to 48 h and then treated with 10 μg/ml Photofrin for 30 min. Excess Photofrin was removed by washing, and cultures were irradiated with a fluence of 18 J/cm^2, while a parallel culture was not irradiated. The metabolic activities of the germ tubes were measured using the XTT assay, and substrate conversion was determined spectrophotometrically at 450 nm. Open bars, irradiated samples. Closed bars, nonirradiated samples.

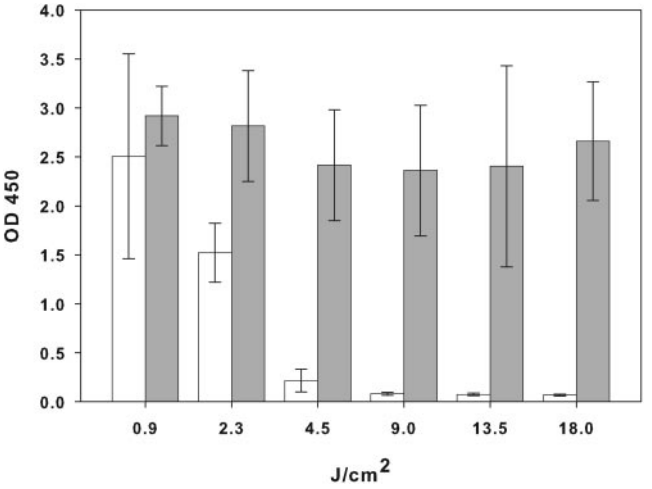

FIG. 6. Photofrin-mediated phototoxicity against intermediate *C. albicans* biofilms as a function of total fluence. *C. albicans* cells were grown in RPMI 1640 supplemented with 1% glucose for 24 h and then treated with 10 μg/ml Photofrin for 30 min. Cultures were irradiated at a fluence rate of 15 mW/cm^2 for durations that resulted in fluences ranging from 0.9 to 18 J/cm^2, while a parallel culture was not irradiated. The metabolic activities of the biofilms were measured using the XTT assay, and substrate conversion was determined spectrophotometrically at 450 nm. Open bars, irradiated samples. Closed bars, nonirradiated samples.

concentration, a third parameter influencing response to photodynamic insult is the fluence of the irradiation applied. Experimental conditions similar to those described for the experiment shown in Fig. 5 were used, and 24-h *C. albicans* biofilms were irradiated over a range of times, resulting in a fluence ranging from 0.9 J/cm^2 to 18 J/cm^2. However, to ensure that the loose upper layer of the biofilm was not excessively damaged during processing, excess Photofrin was not removed by washing prior to irradiation. Hence, the phototoxicities observed also included damage from reactive oxygen intermediates produced at or near the cell surface. Fluence-dependent phototoxic responses were observed (Fig. 6), with significant differences ($P = 0.05$) in organism metabolic activities seen at a fluence of 2.25 J/cm^2 (2.5-min irradiation time) in irradiated cultures compared to nonirradiated cultures.

Comparison of *C. albicans* biofilm metabolic activities after treatment with either Photofrin or amphotericin B. To determine whether conventional antifungal therapy against *C. albicans* biofilms was as effective as photodynamic treatment over a short time period, 24-h biofilms were treated with either Photofrin or amphotericin B as a representative fungicidal agent. Biofilms were incubated with 10 μg/ml Photofrin for 30 min and irradiated with a fluence of 18 J/cm^2. Control cultures were treated identically but not irradiated. In parallel, biofilms were treated with 10 μg/ml amphotericin B for the duration of the treatment process (approximately 90 min) and incubated in the dark. Similar to the protocol described for Fig. 6, both Photofrin and amphotericin B were present in the culture until just before the XTT assay was performed. After a 30-min incubation period following irradiation, Photofrin- and amphotericin B-treated cultures were washed and assayed for metabolic activity using the XTT assay. Biofilms treated with Photofrin and irradiated showed a significant reduction in metabolic activity compared to both Photofrin-treated, nonirradiated biofilms ($P = < 0.001$) and amphotericin B-treated biofilms ($P = 0.009$) (data not shown).

DISCUSSION

Our previous work (3) demonstrated the susceptibility of the fungus *Candida* to the photodynamic effects of Photofrin. In the present studies, we defined conditions of photodynamic treatment that resulted in significant phototoxicity to *C. albicans* while reducing the time of exposure of the organism to Photofrin, minimizing the concentration of Photofrin needed, and minimizing the fluence of irradiation applied to the organism. Similar to efforts directed against the treatment of tumors, the success of PDT against candidiasis in vivo will require maximizing the differential between the phototoxicity against the organism and that against normal host tissue. In tumor systems, this differential is based, in part, on the selective retention of photosensitizing agent by tumor cells compared to that by normal cells.

Exposure times of as short as 1 to 5 min were adequate for the effective treatment against *C. albicans* germ tubes, and the short incubation time did not require a substantially higher drug concentration to exert a significant phototoxic effect. The brief exposure time to Photofrin and the low concentration required to mediate a significant degree of damage to *C. albicans* germ tubes indicate that it may be possible to establish effective PDT treatment regimens for candidiasis that will result in minimal damage to surrounding host tissue and to establish that effective phototoxicity against *C. albicans* can be achieved under conditions comparable to a brief topical application of Photofrin and irradiation in vivo. Although these data strongly suggest that PDT will be useful clinically against *C. albicans*, the complexity of the in vivo environment makes it

difficult to extrapolate directly from in vitro studies the drug and light doses that would be optimal in vivo; it is possible that higher doses may be required for effective PDT. Nonetheless, the in vitro studies described here will provide guidelines for testing in animal models of mucocutaneous (28) and cutaneous (13) candidiasis.

Similar to what is seen for other microbes, two mechanisms contributing to drug resistance in *Candida* and other fungi are the enzymatic alteration of the active agent and the induction of drug efflux pumps (35, 41). Therefore, we sought to determine whether organisms preloaded with Photofrin lost photosensitivity over time, which would suggest either the degradation or the efflux of the photosensitizer. Incubation of preloaded organisms for up to 2 h in a hydrophilic environment of either buffer or tissue culture medium supplemented with glucose prior to irradiation did not diminish the phototoxic effect of 10 μg/ml of Photofrin (Fig. 3A and B). The retention of photosensitivity in the absence of serum and the gradual loss of photosensitivity in the presence of serum suggest that *C. albicans* germ tubes do not extensively modify the photosensitizing properties of Photofrin, nor do they rapidly pump the photosensitizer from the cell. Rather, the presence of serum in the incubation medium may result in the gradual leaching of Photofrin to the external milieu, similar to what is seen in tumor cells (1, 24).

In many cell types, including *C. albicans*, exposure to sublethal concentrations of hydrogen peroxide or superoxide anion results in the acquisition of resistance to more toxic concentrations of these oxidative species. This is largely due to the induction of enzymes capable of neutralizing these oxidative species: catalase and superoxide dismutase, respectively (15, 19, 33). The primary oxidative species generated by the interaction of Photofrin, oxygen, and light is singlet oxygen (40). A number of biomolecules, including thioredoxin (7), histidine (32), vitamin B_6 (2), vasoactive intestinal peptide (30), and melatonin (22), are capable of scavenging or quenching singlet oxygen. However, a search of the literature database uncovered no reports of an inducible enzymatic scavenger of singlet oxygen. Furthermore, resistance to singlet oxygen is rare in biological systems, with only the *Cercospora* fungi, a group of plant pathogens, reported as being tolerant to photosensitizers that generate singlet oxygen. Resistance to singlet oxygen is under the control of the *SOR1* gene in *Cercospora*, and orthologs are found in a wide range of species, including yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) (11, 12). Two *Cercospora* genes involved in singlet oxygen detoxification include a multidrug ABC transporter protein and a member of the pyridine nucleotide-disulfide oxidoreductase family (39). Therefore, we reasoned that an as-yet-undiscovered inducible mechanism of resistance to singlet oxygen could be expressed in *C. albicans*.

Since *C. albicans* produces catalase, we confirmed previous observations that resistance to subsequent H_2O_2 challenge could be induced in germ tubes after initial exposure to subtoxic levels of H_2O_2. In contrast to this observation, primary treatment of *C. albicans* germ tubes with a subtoxic concentration of Photofrin did not result in a comparable acquisition of resistance to increasing concentrations of Photofrin in a secondary treatment. Hence, no adaptation to a primary singlet oxygen stress similar to that seen with hydrogen peroxide was observed in *C. albicans* germ tubes. Furthermore, no cross talk was observed in the responses of *C. albican*s to the two different treatments used in sequence, regardless of the order of administration. These data suggest that reactive oxygen intermediates produced by the photodynamic activation of Photofrin internalized by *C. albicans* does not include an amount of hydrogen peroxide sufficient to induce significant catalase production; the data also demonstrate that the response to oxidative stress induced by H_2O_2 does not elicit protection against subsequent challenge with Photofrin.

Both in the environment and during the course of infection, organisms frequently exist in adherent, organized communities termed biofilms rather than as the independent entities usually seen during planktonic growth (37). Of particular importance clinically, biofilm populations are more resistant to antibiotic concentrations that are effective against the same population if the biofilm is dispersed (14, 16, 18). We sought to determine whether biofilm development rendered *C. albicans* more resistant to Photofrin-mediated phototoxicity.

Under the experimental conditions used, the sensitivity of *C. albicans* to Photofrin-mediated phototoxicity did not diminish during the course of mature (48-h) biofilm formation (Fig. 5). Attempts to examine photodynamic efficacy against mature *C. albicans* biofilms over longer time periods of development were thwarted by the fragility of the older biofilms, which did not hold up well to the necessary wash steps. Intermediate-stage biofilms proved to be fairly resilient to manipulation and were used in subsequent experiments. The intermediate-stage (24-h) *C. albicans* biofilms were as sensitive as germ tubes to Photofrin-mediated phototoxicity in terms of the fluence of irradiation applied.

Intermediate biofilms treated with a high concentration of amphotericin B (10 μg/ml) over the time period required to complete the photosensitization and irradiation protocol against parallel cultures did not show a significant reduction in XTT metabolism compared to Photofrin-treated, nonirradiated cultures. Treatment of planktonic *C. albicans* blastoconidia with 1 to 8 μg/ml of amphotericin B for a comparable time period significantly reduced the plating efficiency of the organism (26), demonstrating that, under the proper conditions, amphotericin B can exert a toxic effect in a relatively brief time frame. In contrast to the sensitivity of planktonic cells to both fluconazole and amphotericin B, Kuhn et al. (25) demonstrated increased resistance of *C. albicans* biofilms to both the azole and the free amphotericin B, as measured by the XTT assay. In the same study, only liposome-encapsulated amphotericin B and the cell wall synthesis inhibitor echinocandin (25) displayed efficacies against *C. albicans* biofilms. Thus, Photofrin-mediated phototoxicity against *C. albicans* biofilms in vitro appeared at least comparable to the most effective antifungal formulations in current use.

We have begun to define conditions for effective photodynamic treatment against *C. albicans* in vitro that will serve as guidelines for investigating the efficacy of Photofrin in PDT for candidiasis in experimental models of superficial infection. It is difficult to mimic the complex milieu of a cutaneous or mucocutaneous infection in vitro. However, knowing the lower limits of photosensitizer exposure time, photosensitizer concentration, and total fluence required to mediate significant damage against the organism in vitro will allow for the rational

design of animal studies similar to those described by Teichert et al. (38), who used methylene blue in PDT of experimental oral candidiasis. Using these in vitro parameters of effective photosensitization as a baseline, we have also shown that several of the mechanisms that microorganisms use to subvert either antimicrobial oxidative defenses or antimicrobial therapy are apparently not operative during Photofrin-mediated phototoxicity of *C. albicans*. These observations provide support and rationale for the continued investigation of PDT as an adjunctive, or possibly alternative, mode of therapy against cutaneous and mucocutaneous candidiasis.

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