Blue Dye and Red Light, a Dynamic Combination for Prophylaxis and Treatment of Cutaneous *Candida albicans* Infections in Mice[▽]

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The objective of this study was to investigate photodynamic therapy (PDT), using blue dye and red light, for prophylaxis and treatment of cutaneous *Candida albicans* infections in mice. A mouse model of skin abrasion infected with *C. albicans* was developed by inoculating wounds measuring 1.2 cm by 1.2 cm with 10⁶ or 10⁷ CFU. The use of a luciferase-expressing strain of *C. albicans* allowed real-time monitoring of the extent of infection in mice noninvasively through bioluminescence imaging. The phenothiazinium salts toluidine blue O (TBO), methylene blue (MB), and new methylene blue (NMB) were compared as photosensitizers (PS) for the photodynamic inactivation of *C. albicans* *in vitro*. PDT *in vivo* was initiated either at 30 min or at 24 h after fungal inoculation to investigate the efficacies of PDT for both prophylaxis and treatment of infections. Light at 635 ± 15 nm or 660 ± 15 nm was delivered with a light dose of 78 J/cm² (for PDT at 30 min postinfection) or 120 J/cm² (for PDT at 24 h postinfection) in multiple exposures with bioluminescence imaging taking place after each exposure of light. *In vitro* studies showed that NMB was superior to TBO and MB as the PS in the photodynamic inactivation of *C. albicans*. The efficacy of PDT was related to the ratio of PS concentration to fungal cell density. PDT *in vivo* initiated either at 30 min or at 24 h postinfection significantly reduced *C. albicans* burden in the infected mouse skin abrasion wounds. These data suggest that PDT is a viable approach for prophylaxis and treatment of cutaneous *C. albicans* infections.

Superficial fungal skin infection is one of the most frequent diseases, affecting millions of people worldwide (13, 17). Although rarely life threatening, these infections can have debilitating effects on a person's quality of life and may in some circumstances become invasive. The most frequently encountered fungal pathogen is *Candida albicans* (13, 17, 30). With the steadily increasing incidence of fungal infections over the last several decades, the number of effective antifungal drugs remains limited (3). Topical antifungal drugs, such as clotrimazole, are the options for cutaneous *C. albicans* infections. However, resistance of *C. albicans* to clotrimazole has been reported (23, 24), and furthermore, the incidence of resistance to antifungal drugs may be increasing (16, 29). As a result, there is a pressing need for the development of new antifungal treatment modalities.

Photodynamic therapy (PDT) is based on the concept that a nontoxic dye, known as a photosensitizer (PS), can be localized preferentially in certain tissues or cells and subsequently activated by harmless visible light to produce reactive oxygen species (ROS) that can kill the cells that have bound the PS. Numerous published studies have shown PDT to be highly effective in the *in vitro* inactivation of fungi (19, 20, 31). In addition, it is considered that the development of resistance to PDT by microbes is an unlikely event, because PDT is typically

a multitarget process, which is a difference between PDT and most other antifungal drugs (22).

In the present study, we investigated the efficacy of PDT using a combination of blue dye (phenothiazinium salt) and red light for the prophylaxis and treatment of *C. albicans* infection in mouse skin abrasion wounds. It is known that phenothiazinium salts can localize in the plasma membrane of fungal cells (9) and have been proven to be effectively phototoxic *in vitro* to fungal cells (11, 14, 27). The use of a luciferase-expressing strain of *C. albicans* allowed the real-time monitoring of the extent of infection in mice noninvasively through a bioluminescence imaging technique.

MATERIALS AND METHODS

Photosensitizers. The phenothiazinium salts toluidine blue O (TBO) (T3260-5G; Sigma, St. Louis, MO), methylene blue (MB) (M9140-25G; Sigma), and new methylene blue (NMB) (556416-1G; Sigma) were used as the PSs in the study. The dye contents of TBO, MB, and NMB were approximately 82%, 85%, and 70%, respectively. Stock PS solutions (1 mM) were prepared by dissolution of the PS powders in phosphate-buffered saline (PBS) and stored in the dark at 4°C for no longer than 14 days.

Light source. Light was delivered topically using a noncoherent light source (LumaCare, Newport Beach, CA) that provides the full spectrum of visible light (400 nm to 800 nm). The system employed interchangeable filter probes and produced a uniform spot for illumination. We used a 635 ± 15-nm filter probe for TBO- and NMB-mediated PDT and a 660 ± 15-nm filter probe for MB-mediated PDT. The emission spectra of the filter probes were measured using a spectroradiometer (SPR-01; Luzchem Research Inc., Ottawa, ON, Canada) and are shown in Fig. 1 together with the absorption spectra of the dyes, which were measured using an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific, San Jose, CA). The light power was routinely measured using a laser power meter (Fieldmate; Coherent, Portland, OR).

Cells and culture conditions. The luciferase-expressing *C. albicans* strain used in this study was CEC 749 (10). The luciferase reporter was constructed by fusing

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FIG. 1. (A) Structures of the phenothiazinium salt photosensitizers MB, TBO, and NMB. (B) Visible absorption spectra of NMB, MB, and TBO (10 μ M in water). (C) Emission spectra of the LumaCare 635-nm and 660-nm filter probes.

a synthetic, codon-optimized version of the *Gaussia princeps* luciferase gene (gLuc59) to *C. albicans* PGA59, which encodes a glycosylphosphatidylinositol-linked cell wall protein. Luciferase expressed from this PGA59-gLuc fusion was localized at the *C. albicans* cell surface (10), where it can easily come into contact with its substrate coelenterazine in intact cells. This system allows bioluminescence imaging of *C. albicans* infections after simple addition of coelenterazine solution to the surface of the infected tissue (10). This approach also allowed the detection of luciferase in both hyphae and yeast cells (8, 10), given the central importance of the yeast-hypha transition in *C. albicans* virulence (1).

C. albicans was routinely grown at 30°C on yeast extract-peptone-dextrose (YPD) agar and subcultured in YPD medium. The inoculum was prepared by growing the *C. albicans* cells in 3 ml YPD broth overnight at 30°C with shaking at 130 rpm in a C24 incubator/shaker (New Brunswick Scientific, Edison, NJ). The broths were then centrifuged at 12,000 rpm for 2 min (centrifuge 5417 C; Eppendorf, Hamburg, Germany) and resuspended in 0.5 ml PBS. The concentrations were then adjusted to the appropriate values for the experiment by measuring the optical density at 570 nm (OD_{570}) of the *C. albicans* suspensions, using an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific). An OD_{570} of 0.65 corresponded to a fungal concentration of 10^7 CFU/ml in PBS.

Mouse model of skin abrasion infection. All animal procedures in this study were approved by the Subcommittee on Research Animal Care (IACUC) of the Massachusetts General Hospital (protocol number 2005N000111) and were in accordance with the guidelines of the National Institutes of Health (NIH). Adult female BALB/c mice (Charles River Laboratories, Wilmington, MA), 7 to 8 weeks old and weighing 17 to 21 g, were used in all experiments. To prevent fighting and mutual interference with the wounds, the animals were housed one per cage with access to food and water *ad libitum* and were maintained on a 12-h light/dark cycle under a room temperature of 21°C.

Before the creation of wounds, the mice were anesthetized by intraperitoneal (i.p.) injection of a ketamine-xylazine cocktail and then shaved on the dorsal surfaces. Mouse skin was scraped with no. 15 scalpel blades (08-927-5C; Fisher Scientific, Agawam, MA) until a reddened area appeared (just short of drawing blood). Each wound measured approximately 1.2 cm by 1.2 cm. With a pipette tip, the surface of each wound was inoculated with one drop (40 μ l) of the prepared culture suspension containing 10^6 or 10^7 CFU of *C. albicans*, which was smeared onto the wound surface with an inoculating loop.

Bioluminescence imaging. The setup consisted of a charge-coupled-device (CCD) camera (model C2400-30H; Hamamatsu Photonics, Bridgewater, NJ), a camera controller, a light-tight imaging chamber, an image processor (C5510-50; Hamamatsu), and a color monitor (PVM 1454Q; Hamamatsu). Light-emitting diodes (LEDs) are mounted inside the imaging chamber and supply the light required for obtaining a grayscale image of the sample. Using photon counting mode, an image can be obtained by detecting and integrating individual photons emitted by the fungal cells.

Prior to imaging, mice were anesthetized by i.p. injections of ketamine-xylazine cocktail. Twenty microliters of coelenterazine (500 μ g/ml in 1:9 methanol-PBS; Gold Biotechnology, Inc., St. Louis, MO) was topically smeared onto the surface of each infected wound. Mice were then placed on an adjustable stage in the imaging chamber, and the infected wounds were positioned directly under the camera. The concentration of coelenterazine was chosen based on a published protocol (10). The 10% methanol present in the solvent was shown to not be toxic to the *Candida* cells. A grayscale background image of each wound was made, and this was followed by a bioluminescence image of the same region displayed in a false-color scale ranging from pink (most intense) to blue (least intense) and superimposed on the grayscale image. The signal from the bioluminescence image was quantified as relative luminescence units (RLUs) using Argus software (Hamamatsu).

Photodynamic inactivation of *C. albicans* in vitro. A 3-ml *C. albicans* suspension containing 10^7 CFU/ml in PBS was placed into a 35-mm petri dish to give a depth of 3 mm liquid and incubated in the dark with 20 μ M dye (TBO, NMB, or MB) for 30 min at room temperature. The dye concentration was chosen by a preliminary study based on the criterion of effective photodynamic inactivation with minimal dark toxicity. The suspension then was irradiated with the LumaCare lamp at an irradiance of 32.5 mW/cm² with the lid of the petri dish removed. The 635 $\pm$ 15-nm filter probe was used for TBO- and NMB-mediated PDT and the 660 $\pm$ 15-nm filter probe for MB-mediated PDT. During the irradiation, the *C. albicans* suspension was stirred by a minimagnetic bar (Fisher Scientific Co., Norcross, GA). Aliquots of 50 μ l of the fungal suspension were withdrawn at 0, 1, 2, 3, 4, and 5 min (corresponding to delivered light doses of 0, 1.95, 3.90, 5.85, 7.80, and 9.75 J/cm², respectively). Post-PDT CFU were then determined by serial dilution on YPD agar plates using the method of Jett et al.

FIG. 2. Linear correlation between fungal bioluminescence (relative light units) and CFU. Experiments were performed in six replicates. Data are means, and bars represent standard deviations.

(18). Colonies were allowed to grow for 24 to 48 h at 30°C. Experiments were performed in triplicate for each PS.

PDT *in vivo* for mouse skin abrasions infected with *C. albicans*. PDT was initiated either at 30 min or at 24 h after fungal inoculation to investigate the efficacies of PDT for both prophylaxis and treatment of infections. Inocula of 10^6 and 10^7 CFU were applied to groups of wounded mice ($n = 11$) that were then treated with PDT at 30 min and at 24 h after fungal inoculation. Before light irradiation, 50 μ l NMB solution (400 μ M PS equivalent) was smeared onto each wound, and the wound and PS were then incubated for 30 min in the dark. The dye concentration for the *in vivo* study was also selected by a preliminary study based on the criterion that the concentration rendered an effective PDT with minimal dark toxicity. The *in vivo* dark toxicity of the dye was quantified by bioluminescence imaging. Mice were given a total light exposure of up to 78 J/cm² (for PDT at 30 min postinfection) or 120 J/cm² (for PDT at 24 h postinfection) in multiple exposures, with bioluminescence imaging taking place after each exposure of light. The light was delivered at an irradiance of 32.5 mW/cm². To record the time course of the extent of fungal infection, fungal luminescence from the mouse wounds was measured daily after PDT until the infections were cured (disappearance of fungal luminescence) or the wounds were healed.

To correlate the fungal luminescence intensity with the viability of *C. albicans in vivo* after PDT, mouse wounds were surgically excised and homogenized in 10 ml PBS with a tissue homogenizer (Omni International, Kennesaw, GA). The suspensions contained a mixture of tissue and *C. albicans* and were subjected to 5 serial 10-fold dilutions in PBS and then streaked on square YPD agar plates for CFU counting after 24 to 48 h of incubation at 30°C.

Statistical analysis. In a two-dimensional coordinate system, the area under the curve (AUC) data, which represent the time courses of fungal luminescence of the infected wounds and also the overall fungal burden of the infected wounds, were calculated using numerical integration (5). Differences in AUC between the untreated control and the PDT-treated groups and between different PDT-treated groups were compared for statistical significance using one-way analysis of variance (ANOVA). *P* values of <0.05 were considered significant.

RESULTS

Fungal luminescence intensity linearly correlated with the corresponding *C. albicans* CFU. We compared side by side the *C. albicans* CFU using a colony-forming assay and the corresponding fungal luminescence from the suspensions at different cell densities using a bioluminescence imaging technique. Figure 2 demonstrates a perfect linear correlation ($R^2 = 0.999991$, $P = 1.011 \times 10^{-8}$) between the fungal luminescence and the corresponding *C. albicans* CFU.

NMB was superior to MB and TBO as the PS in inactivating *C. albicans in vitro*. Results for the photodynamic inactivation of *C. albicans in vitro* at a cell density of 10^7 CFU/ml using NMB, MB, or TBO as the PS are shown in Fig. 3. When a 9.75-J/cm² light dose had been delivered, approximately 4.43 log₁₀ CFU inactivation was achieved when NMB was used as

FIG. 3. Light dose-dependent photodynamic inactivation of *C. albicans* (10^7 cells/ml) *in vitro* using 20 μ M NMB, MB, or TBO and a 30-min incubation. Experiments were performed in triplicate. Data are means, and bars represent standard deviations.

the PS. In contrast, almost no or only modest fungal inactivation was observed under the equivalent conditions when MB or TBO was used. As a result, NMB was selected as the PS in the following studies.

Loss of fungal luminescence (RLU) did not linearly correlate with loss of CFU during the course of PDT. We compared the *in vitro* PDT dose response of reduction of fungal luminescence with the reduction of the corresponding fungal CFU (Fig. 4). The remaining fraction of fungal luminescence with increasing light doses of PDT was significantly lower than the remaining fraction of CFU at low light doses of PDT until the light dose of PDT reached approximately 7.8 J/cm². We believe that this finding reflects the localization of the luciferase enzyme on the surface of the *C. albicans* cells, where it can be damaged by the ROS produced during PDT, allowing loss of bioluminescence signal without corresponding killing of the cells. However, when the light dose was further increased, the remaining fraction of fungal bioluminescence became higher than that of fungal CFU, i.e., the curves crossed over. As a matter of fact, after the fungal luminescence decreased approximately 2.5 log₁₀ from its initial value, the rate of decrease of fungal luminescence

FIG. 4. *In vitro* light dose responses of fungal luminescence and corresponding fungal CFU. Experiments were performed in triplicate. Data are means, and bars represent standard deviations.

FIG. 5. (A) Successive bioluminescence images of a representative mouse skin abrasion infected with 10^7 CFU *C. albicans*. (B and C) Representative periodic acid-Schiff-stained skin biopsy specimen taken from a mouse skin abrasion infected with 10^7 CFU *C. albicans*, showing the presence of yeasts (B, arrows), hyphal filaments (C, circle), and inflammatory infiltrate (C). Biopsy was taken on day 1 postinfection. Bar, 20 μ m.

leveled off and was lower than the rate of decrease of CFU. This can be explained by the sensitivity limitation of the photon-counting camera at low photon fluxes.

Mouse model of skin abrasion infected with *C. albicans* showed that stable infections developed, as demonstrated by bioluminescence imaging and histopathological examination. Figure 5A shows the successive bioluminescence images from day 0 to day 5 of a representative mouse skin abrasion infected with 10^7 CFU *C. albicans*. The fungal luminescence intensity peaked on day 1 (as quantified by the analysis of fungal luminescence image) and remained strong until day 3. The scab of the wound detached from the mouse body on day 5, and only modest fungal luminescence remained after the detachment of the wound scab.

A representative periodic acid-Schiff (PAS)-stained skin biopsy specimen taken from an infected wound on day 1 postinfection demonstrated the presence of numerous *C. albicans* cells in the form of yeast (Fig. 5B, arrows) and multicellular hyphal filaments (Fig. 5C, circle). While all yeast cells were located on the surface of the wound, the hyphal filaments branched out in every direction into the tissue. A robust host response to *C. albicans* infection, characterized by an inflammatory infiltrate, was also observed (Fig. 5C).

Efficacy of PDT was related to the ratio of dye concentration to fungal cell density. We found that the efficacy of PDT was related to the ratio of dye concentration to *C. albicans* cell density. As we reported in the *in vitro* studies described above, when the initial average *C. albicans* cell density was 10^7 CFU/ml and the NMB concentration was 20 μ M, approximately 4.43 log CFU inactivation was achieved after 9.75 J/cm² light had been delivered. However, when we used 10^8 CFU/ml and 20 μ M NMB, the surviving fraction of *C. albicans* was only approximately 70% under the same light dose (data not shown). When we increased the NMB concentration 10-fold (to 200 μ M) and kept the *C. albicans* cell density at 10^8 CFU/ml, the remaining fraction of *C. albicans* was still only approximately 39% after 9.75 J/cm² light had been delivered. We believe that higher cell densities led to a lower NMB concentration per cell, while an attempt to compensate for this limited availability of NMB by using a higher dye concentration resulted in self-quenching of the dye and more attenuation of

light penetration into the microbial suspension and subsequently reduced the efficacy of PDT.

PDT effectively prevented or treated *C. albicans* skin abrasion infections in mice. We first compared the difference in PDT efficacy *in vivo* for two fungal inocula of 10^6 CFU and 10^7 CFU. Figure 6A displays the set of bioluminescence images of a representative mouse skin abrasion wound infected with 10^6 CFU *C. albicans* and treated with PDT. PDT, with the combination of 400 μ M NMB and 78.0 J/cm² red light, was carried out 30 min after the fungal inoculation. Only a few pixels of remaining fungal luminescence could be observed immediately after PDT, and no significant reoccurrence of infection (as indicated by bioluminescence imaging) was observed at 24 h (day 1) after PDT. Figure 6B is a representative PAS-stained skin biopsy specimen taken from a skin abrasion wound infected with 10^6 CFU and treated with PDT on day 0. Biopsy was taken at 24 h after PDT. Only a few yeast cells could be observed in the histological section (Fig. 6B, ovals). The histology also demonstrated the presence of numerous inflammatory cells in the skin. Figure 6C is the set of bioluminescence images of a representative mouse skin abrasion infected with 10^6 CFU *C. albicans*, applied with 400 μ M NMB but without light illumination (dark control). Almost no fungal luminescence reduction was observed for the dark control over a time period equivalent to that for PDT treatment (Fig. 6C).

Figure 6D shows the set of bioluminescence images of a representative mouse skin abrasion wound infected with 10^7 *C. albicans* CFU and treated with PDT on day 1, with the same parameters as those used above for the 10^6 CFU *C. albicans* inoculum. The PDT dose response of fungal luminescence with the 10^7 CFU inoculum was similar to that with the 10^6 CFU inoculum, and also only a few pixels of remaining fungal luminescence could be observed immediately after PDT. However, in the case of the 10^7 CFU fungal inoculum, pronounced reoccurrence of fungal luminescence (RLU) was found in the mouse at 24 h after PDT. We further compared the *in vivo* PDT dose response of fungal luminescence with that of the corresponding CFU of the mouse skin abrasions infected with 10^7 CFU *C. albicans* and treated with PDT at day 0. CFU of the infected mouse skin abrasions ($n = 3$) was quantified by homogenizing the mouse skin abrasions in PBS followed by

FIG. 6. (A) Successive bioluminescence images of a representative mouse skin abrasion infected with 10^6 CFU *C. albicans* and treated with PDT at 30 min after fungal inoculation. PDT was carried out with a combination of 400 μ M NMB and 78 J/cm² red light. (B) Periodic acid-Schiff-stained skin biopsy specimen taken from the *C. albicans*-infected mouse skin abrasion shown in panel A. The biopsy specimen was taken on day 1 postinfection/PDT. Ovals, yeasts. Bar, 20 μ m. (C) Successive bioluminescence images of a representative mouse skin abrasion infected with 10^6 CFU *C. albicans*, applied with 400 μ M NMB but without red light illumination (dark control). (D) Successive bioluminescence images of a representative mouse skin abrasion infected with 10^7 CFU *C. albicans* and treated with PDT at 30 min after fungal inoculation. PDT was carried out with a combination of 400 μ M NMB and 78 J/cm² red light. (E) Mean remaining fractions of fungal luminescence and corresponding CFU of mouse skin abrasions ($n = 3$) infected with 10^7 CFU *C. albicans* and treated with PDT at 30 min after fungal inoculation. Bars represent standard deviations. (F) Time courses of mean fungal luminescence of the infected mouse skin abrasions treated with PDT on day 0 (10^6 CFU *C. albicans* inoculum, PDT at 30 min after fungal inoculation, light dose of 78 J/cm², $n = 11$) or not treated (10^6 CFU *C. albicans* inoculum, $n = 11$). Bars represent standard deviations. (G) Mean areas under the bioluminescence-time curves (in the two-dimensional coordinate system shown in panel F), representing the overall fungal burden of the mouse wounds in different groups of mice (PDT versus no treatment, $P = 0.0055$). Bars represent standard deviations. (H) Time courses of mean fungal luminescence of the infected mouse skin abrasions treated with PDT on day 0 (10^7 CFU *C. albicans* inoculum, PDT at 30 min after fungal inoculation, light dose of 78 J/cm², $n = 9$) or not treated (10^7 CFU *C. albicans* inoculum, $n = 11$). Bars represent standard deviations. (I) Mean areas under the bioluminescence-time curves (in the two-dimensional coordinate system shown in panel H), representing the overall fungal burden of the mouse wounds in different groups of mice (PDT versus no treatment, $P = 0.011$). Bars represent standard deviations.

plating and counting. It can be seen from Fig. 6E that a loss of $2.7 \log_{10}$ fungal luminescence resulting from PDT actually corresponded to only a $0.77 \log_{10}$ loss of CFU ($P = 0.003$). Taken together with the *in vitro* observations (Fig. 6), we confirmed that a sufficient fraction of *C. albicans* was not inactivated after PDT when an inoculum of 10^7 CFU was used and that a fraction of the *C. albicans* cells just temporarily lost their bioluminescence due to PDT inactivation of the luciferase enzyme. Another possibility of the fungal regrowth is that the hyphae located in the dermis regrew at the surface of the wound, which would be indicative that the penetration of NMB into the wound bed was limited.

Figure 6F shows the time courses of the mean fungal luminescence values (RLU) from day 0 to day 5 of the mouse skin abrasions infected with 10^6 CFU *C. albicans* and treated with PDT on day 0 (i.e., 30 min after fungal inoculation) or not treated. Statistical comparison of the areas under the curves (AUC) of fungal luminescence time course in the two-dimensional coordinate system shown in Fig. 6F demonstrated that PDT significantly reduced the fungal burden of the infected wounds by 95.4% ($1.34 \log_{10}$) (Fig. 6G). The mean AUC values for the PDT-treated mice and nontreated mice were 1.54×10^5 and 3.30×10^6 , respectively, indicating a reduction of 95.3% in fungal burden as a result of PDT ($P = 0.0055$).

Figure 6H shows the time courses of the mean fungal luminescence values from day 0 to day 5 of the mouse skin abrasions infected with 10^7 CFU *C. albicans* and treated with PDT on day 0 or not treated. An increase of fungal luminescence can be observed from day 0 after PDT to day 1. The mean AUC values for the PDT-treated mice and nontreated mice were 1.66×10^6 and 6.66×10^6 (Fig. 6I), respectively, indicating a reduction of approximately 75.0% ($0.60 \log_{10}$) in fungal burden resulting from PDT ($P = 0.011$).

To assess the efficacy of PDT on established infections, we also performed experiments on the mouse skin abrasions infected with 10^7 CFU *C. albicans* and treated with PDT on day 1 (24 h after fungal inoculation) with the combination of 400 μ M NMB and a red light dose of 120 J/cm². Figure 7A shows the time courses of the mean fungal luminescence values from day 0 to day 5 of the mouse skin abrasions infected with 10^7 CFU *C. albicans* and treated with PDT on day 1 or not treated. The mean AUC values of the PDT-treated mice and nontreated mice were, respectively, 1.74×10^5 and 6.66×10^6 , a reduction of 97.4% in fungal burden as a result of PDT (Fig. 7B). It can be seen that in contrast to PDT of infections produced by 10^7 CFU *C. albicans* and treated on day 0, which led to recurrence of the infection (see above), when an infection caused by the same inoculum (10^7 CFU) was treated on day 1 there was no detectable recurrence of infection.

DISCUSSION

The data obtained from the present study demonstrated that PDT, with the combination of blue dye (NMB) and red light, can significantly reduce the fungal burden in skin abrasion wounds infected with *C. albicans*. Skin abrasion infections are superficial in nature and can be considered suitable for treatment by PDT, as penetration of both the dye (NMB) and the light into tissue is somewhat limited (9).

We found that NMB was much more effective than either

FIG. 7. (A) Time courses of mean fungal luminescence of the infected mouse skin abrasions treated with PDT on day 1 (10^7 CFU *C. albicans* inoculum, PDT at 24 h after fungal inoculation, light dose of 120 J/cm², $n = 11$) or not treated (10^7 CFU *C. albicans* inoculum, $n = 11$). Bars represent standard deviations. (B) Mean areas under the bioluminescence-time curves in the two-dimensional coordinate system shown in panel A (PDT versus no treatment, $P = 0.002$). Bars represent standard deviations.

MB or TBO for the photodynamic inactivation of *C. albicans*. Wainwright et al. (26, 35, 36) reported in their *in vitro* studies that dimethyl methylene blue (DMMB) and NMB are more phototoxic than MB and TBO to both Gram-positive and Gram-negative bacteria and also to cancer cells. Ragas et al. (28) showed that NMB was more effective than MB and TBO against the Gram-negative organism *Acinetobacter baumannii*. To our knowledge, the relative superiority of NMB (or DMMB) against fungi, such as *C. albicans*, has not been reported. The improved activity of NMB is probably due to its more lipophilic nature ($\log P = 1.2$ for NMB), while TBO ($\log P = -0.21$) and MB ($\log P = -0.10$) are considered to be hydrophilic (25, 34).

It was found in the present study that the efficacy of PDT is related to the ratio of dye concentration to fungal cell density. For a high density of *C. albicans* cells, a high concentration of PS and probably a high light dose would be needed for a therapeutically efficient PDT effect. This effect has previously been reported *in vitro*, where it was demonstrated that microbial cells compete for PS molecules and that a high cell density reduces the number of PS molecules available per cell (7). However, too high a concentration of PS will significantly attenuate the light penetrating into the infected wounds by fruitlessly absorbing the light by dye molecules not bound to any microbial cell and therefore impair the efficacy of PDT.

We previously carried out studies (4, 6, 12, 15, 21) using bioluminescent bacterial strains to monitor in real time the viability of bacteria during the course of PDT for *in vivo* infections. These bacterial strains were rendered bioluminescent by stably transforming the entire *Photorhabdus luminescens lux* operon into the bacterial cells. Photodynamic inactivation of microorganisms is intended to destroy their cell membranes, which subsequently leads to the leakage of intracellular materials, including the luciferase substrate (decanal) and the energy source for the reaction (NADH). As a result, the loss of luminescence of the abovementioned luminescent strains of bacteria directly indicates the loss of viability of the bacterial cells. For the *C. albicans* strain we used in this study, luciferase was localized at the plasma membrane because the substrate coelenterazine is not taken up into *C. albicans* cells (10). Due to the location of the enzyme, it can be damaged by the ROS generated during PDT before the fungal cell wall is actually damaged by PDT. Loss of luminescence of this strain of *C. albicans*, therefore, does not absolutely parallel the loss of cell viability during the course of PDT. This observation is confirmed by both the *in vitro* and the *in vivo* tests in the present study. On the other hand, our *in vitro* study also showed that, when there was no external intervention (e.g., PDT), there existed a perfect linear correlation between the fungal luminescence and the corresponding CFU. Accordingly, the fungal luminescence can still serve as the quantitative measurement of the extent of infection, for example, after PDT.

In the present study, for PDT carried out at 30 min after fungal inoculation, each mouse wound was inoculated with 10^6 or 10^7 CFU *C. albicans*. Regrowth was observed with the larger inoculum but was not seen with the smaller one despite the similarity in immediate effect of the PDT in reducing the bioluminescence signal. Interestingly, when PDT was carried out on established infections 24 h after addition of the larger inoculum (10^7 CFU) to the wounds, we observed not only a good reduction of bioluminescence but also no regrowth. Although a higher dose of light was delivered for the established infections, this was still somewhat surprising since it might have been supposed that established infections would be harder to treat than recent contaminations. It is possible that in the fresh wounds there is a higher concentration of protein that has exuded from the wounded tissue and that these protein molecules compete with the *C. albicans* cells for binding the NMB dye. In the infected wounds after 24 h, this protein exudate may have dried up and allowed the NMB a better chance to bind to the *C. albicans* cells. It should be realized that these mouse models with high fungal cell densities are somewhat artificial because clinical wounds are rarely contaminated by such a high number of fungal cells. Even with the large fungal inocula used in the present study, PDT still showed its effectiveness. Therefore, one can expect a better efficacy of PDT for smaller fungal inocula, such as those encountered in clinical situations.

There have been several clinical reports of PDT for fungal infections of the skin (2, 32, 33). Most of these have used topically applied aminolevulinic acid (ALA), which is a precursor of the PS protoporphyrin IX. The high efficacy shown by NMB in the mouse model of *C. albicans* infection suggests that it should be considered for a clinical trial of superficial *C.*

albicans infection, whether in the skin or in the oral or vaginal mucosa.

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We declare no conflict of interest.

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