

## Radioimmunotherapy Is More Effective than Antifungal Treatment in Experimental Cryptococcal Infection

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**Radioimmunotherapy (RIT) prolongs the survival of mice infected with *Cryptococcus neoformans*. To compare the efficacy of RIT with that of amphotericin B, we infected AJ/Cr mice intravenously with either nonmelanized or melanized *C. neoformans* cells. Infected mice were either left untreated or treated 24 h after infection with <sup>213</sup>Bi-18B7 antibody, amphotericin B, or both. Melanization before infection did not increase resistance of *C. neoformans* to RIT in vivo. <sup>213</sup>Bi-18B7 treatment almost completely eliminated colony-forming units from the lung and brain, whereas amphotericin B did not decrease the number of colony-forming units. We conclude that RIT is more effective than amphotericin B against systemic infection with *C. neoformans*.**

*Cryptococcus neoformans* infections cause life-threatening meningoencephalitis in immunocompromised patients, which causes more deaths than tuberculosis among patients with AIDS in the developing world [1]. The inability of immunocompromised individuals to mount an effective immune response to fungal infection reduces the efficacy of standard antifungal therapies, such as amphotericin B or fluconazole [2]. Melanization

of *C. neoformans* occurs in the course of infections, is a virulence factor for this microbe, and reduces the effectiveness of antimicrobials including amphotericin B [3], which adds to the need for novel effective treatments of *C. neoformans* in the setting of immunosuppression.

Radioimmunotherapy (RIT) relies on antibodies to deliver cytotoxic  $\alpha$  or  $\beta$  radiation to tumor cells [4]. The radiolabeled monoclonal antibodies (mAbs) ibritumomab tiuxetan and tositumomab and iodine I 131 tositumomab are approved by the US Food and Drug Administration for the treatment of untreated, refractory, and recurrent lymphomas. Several years ago, we introduced RIT into the realm of infectious diseases, showing prolonged survival in mice systemically infected with *C. neoformans* and treated after infection with a radiolabeled mAb specific for *C. neoformans* polysaccharide capsule [5]. This approach shows little acute hematological or long-term pulmonary toxicity [6], and work has begun to uncover the radiobiological and immune mechanisms of RIT for *C. neoformans* [7, 8]. Here, as a step toward bringing RIT for fungal diseases into clinical practice, we compare the efficacy of RIT with that of amphotericin B against systemic experimental *C. neoformans* infection. We hypothesized that a *C. neoformans*-specific antibody radiolabeled with  $\alpha$ -particle-emitting <sup>213</sup>Bi (bismuth-213) or  $\beta$ -particle-emitting <sup>188</sup>Re (rhenium-188) would be able to kill both melanized and nonmelanized *C. neoformans* cells in vivo better than standard antifungal therapy. We also investigated whether the combination of RIT and amphotericin B treatment would produce different results from either therapy alone.

**Methods.** *C. neoformans* var. *neoformans* strain 24067 (American Type Culture Collection) was grown on Sabouraud agar. Nonmelanized cells were incubated overnight in Sabouraud broth; melanized cells were incubated in a subculture for 3 d in minimal medium with 1 mmol/L L-3,4-dihydroxyphenylalanine. Before infection, cells were washed in phosphate-buffered saline and adjusted by use of a hemocytometer to 10<sup>7</sup> cells per milliliter. The plating efficiency was 55% and 70% for nonmelanized and melanized cells, respectively.

The glucuronoxylomannan-binding murine mAb 18B7 (immunoglobulin G1) is described elsewhere [5]. The isotype-matching control mAb MOPC21 was from obtained from MP Biochemicals. <sup>225</sup>Ac/<sup>213</sup>Bi generators were produced at the Institute for Transuranium Elements (Karlsruhe, Germany). Radiolabeling of mAbs with <sup>213</sup>Bi and with <sup>188</sup>Re eluted from a <sup>188</sup>Re/<sup>188</sup>W generator (Oak Ridge National Laboratory, Oak Ridge, TN) was performed as described elsewhere [5].

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For in vitro RIT with  $^{213}\text{Bi}$  (physical half-life, 46 min),  $10^5$  live melanized or nonmelanized cells were incubated with radiolabeled mAbs (0.2–4.0  $\mu\text{g}/\text{mL}$ ) in the test tubes with agitation for 0.5 h at  $37^\circ\text{C}$ , collected by means of centrifugation, incubated in phosphate-buffered saline at  $37^\circ\text{C}$  for 3 h, treated with Tween 80 (0.5%), triturated 20 times, diluted, and plated for colony-forming units (CFUs). The method for in vitro RIT with  $^{188}\text{Re}$  was the same except that cells were incubated for 48 h at  $4^\circ\text{C}$  after the initial  $37^\circ\text{C}$  incubation, to allow  $^{188}\text{Re}$  (half-life, 17 h) to deliver its radiation dose to the cells. For in vitro amphotericin B experiments,  $10^3$  *C. neoformans* cells were incubated with 0–1.0  $\mu\text{g}/\text{mL}$  amphotericin B as a deoxycholate (Bristol-Myers Squibb) at  $37^\circ\text{C}$  for 2 h, aliquots were plated, and CFUs were counted. Cellular dosimetric calculations were performed as described elsewhere [9]. The experiments were performed twice.

For animal experiments, all procedures followed the guidelines of the Institute for Animal Studies of the Albert Einstein College of Medicine. Melanized or nonmelanized *C. neoformans* cells ( $3 \times 10^5$  cells per mouse) were injected into the tail vein of 6–8-week-old female partially complement-deficient AJ/Cr mice (National Cancer Institute). One day after infection, mice infected with nonmelanized or melanized *C. neoformans* were divided into groups of 5. The mice in each group were either untreated; given intraperitoneally 100  $\mu\text{Ci}$  of  $^{213}\text{Bi}$ -18B7; treated at 24, 48, and 72 h after infection with amphotericin B as a deoxycholate at a dose of 1  $\mu\text{g}$  per gram of body weight; or given both treatments. Mice were monitored for survival, and weights were measured every 3 d. At 60 d after infection, mice were killed and the lungs and brains were plated for CFUs and fixed for histopathological analysis. Tissues were stained with hematoxylin and eosin or Gomori-Grocott methenamine silver (GMS; ScyTek) to detect inflammation and *C. neoformans*, respectively. The remaining lung and brain tissues were used to count the CFUs.

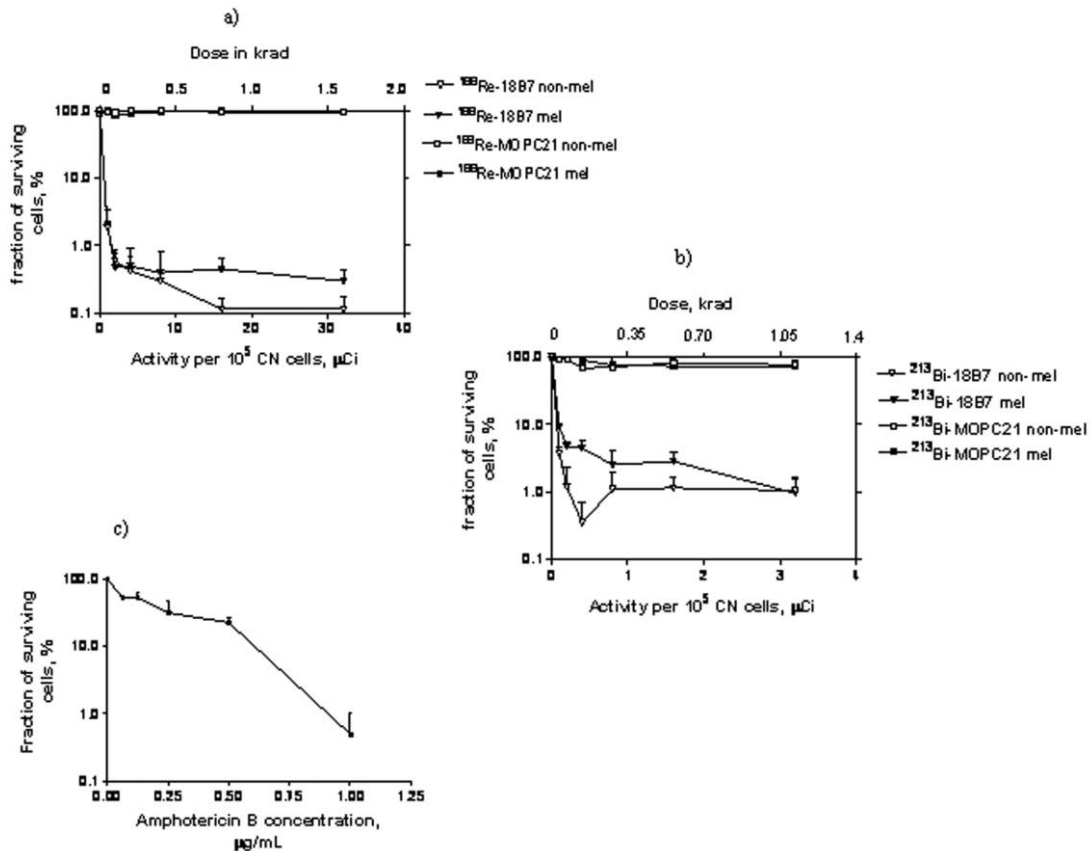
To determine the rate of sterilization of the organs of infected mice by amphotericin B alone, mice were infected with melanized or nonmelanized *C. neoformans* as described above and treated with amphotericin B at a dose of 1  $\mu\text{g}$  per gram of body weight for 14 d. At 7 and 14 d after treatment, 4 mice from each group were killed and the lungs and brains were plated for CFUs. This experiment was performed once. Differences between mice in the number of CFUs and body weight were analyzed with the Student *t* test for unpaired data. Results for which  $P < .05$  were considered significant.

**Results.** Cells of melanized and nonmelanized *C. neoformans* strain 24067 were incubated with  $^{188}\text{Re}$ -18B7 mAb and  $^{213}\text{Bi}$ -18B7 mAb with increasing activity. Incubation of melanized and nonmelanized cells with 1.5  $\mu\text{Ci}$  of  $^{188}\text{Re}$ -18B7 mAb or 0.2  $\mu\text{Ci}$  of  $^{213}\text{Bi}$ -18B7 mAb killed 90% of the cells and delivered cellular radiation doses of 0.1 krad for  $^{188}\text{Re}$ -

18B7 and 0.04 krad for  $^{213}\text{Bi}$ -18B7 (Figures 1A and 1B).  $^{213}\text{Bi}$  or  $^{188}\text{Re}$  conjugated to the irrelevant isotype-matching antibody MOPC21 killed neither type of cell (Figures 1A and 1B). The difference in the susceptibility of melanized and nonmelanized cells to antibody-delivered radiation became obvious when we attempted to achieve 99.9% elimination of cells. Sixteen microcuries (0.8-krad-dose) of  $^{188}\text{Re}$ -18B7 mAb eliminated 99.9% of nonmelanized cells, whereas that degree of cell killing was not achieved for melanized cells in the investigated range of activity.  $^{213}\text{Bi}$ -18B7 mAb killed 99.7% of nonmelanized cells with 0.4  $\mu\text{Ci}$  (0.17 krad-dose), but again that level of cell killing was not observed for melanized cells. As  $\sim 10$  times less  $^{213}\text{Bi}$  radioactivity than  $^{188}\text{Re}$  radioactivity was required to eliminate the bulk of either melanized or nonmelanized cells, we selected  $^{213}\text{Bi}$  mAb for comparison with amphotericin B in vivo. One microgram per milliliter amphotericin B reduced the number of *C. neoformans* CFUs by  $>2$  log units (Figure 1C). Considering the published minimum inhibitory concentration for melanized *C. neoformans* strain 24067 is higher than for that for nonmelanized *C. neoformans* strain 24067 (0.1875 and 0.1250  $\mu\text{g}/\text{mL}$ , respectively), we selected a dose of 1  $\mu\text{g}$  per gram of mouse body weight ( $\sim 17$   $\mu\text{g}$  per mouse, which allowed a transient blood concentration of 8.5  $\mu\text{g}/\text{mL}$ ) for in vivo experiments.

Changes in the mean weights of the AJ/Cr mice in the different treatment groups infected with nonmelanized or melanized *C. neoformans* are shown in Figures 2A and 2B. RIT-treated mice in the nonmelanized *C. neoformans* group experienced some weight loss between days 0 and 20 and then gained weight for the rest of the observation period. RIT-treated mice in the melanized *C. neoformans* group gained weight faster than those in the nonmelanized *C. neoformans* group ( $P = .04$ ). Amphotericin B-treated mice in the nonmelanized *C. neoformans* group lost some weight around day 30 but then recovered (Figure 2A), whereas amphotericin B-treated mice in the melanized *C. neoformans* group gained weight during the whole observation period (Figure 2B). However, the difference in weights between the amphotericin B-treated mice and the RIT-treated mice in the melanized *C. neoformans* group was significant ( $P < .05$ ) only until day 20, until which time the RIT-treated mice were still recovering from RIT-associated weight loss; after day 20, the difference was not statistically significant ( $P > .05$ ). The combined treatment group did best in terms of gaining weight among mice infected with nonmelanized *C. neoformans*, whereas in the melanized *C. neoformans* group, the mice receiving the combination treatment gained more weight than those treated with RIT and then equalized with the RIT-treated mice later in the study.

Analysis of the lungs and brain at 60 d after infection showed that amphotericin B did not significantly decrease the number of CFUs in the lungs and brain of mice in either the non-



**Figure 1.** In vitro killing and dosimetry of melanized and nonmelanized *Cryptococcus neoformans* (CN) cells treated with <sup>188</sup>Re-labeled *C. neoformans* polysaccharide-specific 18B7 and control isotype-matching MOPC21 monoclonal antibodies (A), <sup>213</sup>Bi-labeled *C. neoformans* polysaccharide-specific 18B7 and control isotype-matching MOPC21 monoclonal antibodies (B), or amphotericin B (C). Mel, melanized *C. neoformans* cells; non-mel, nonmelanized *C. neoformans* cells.

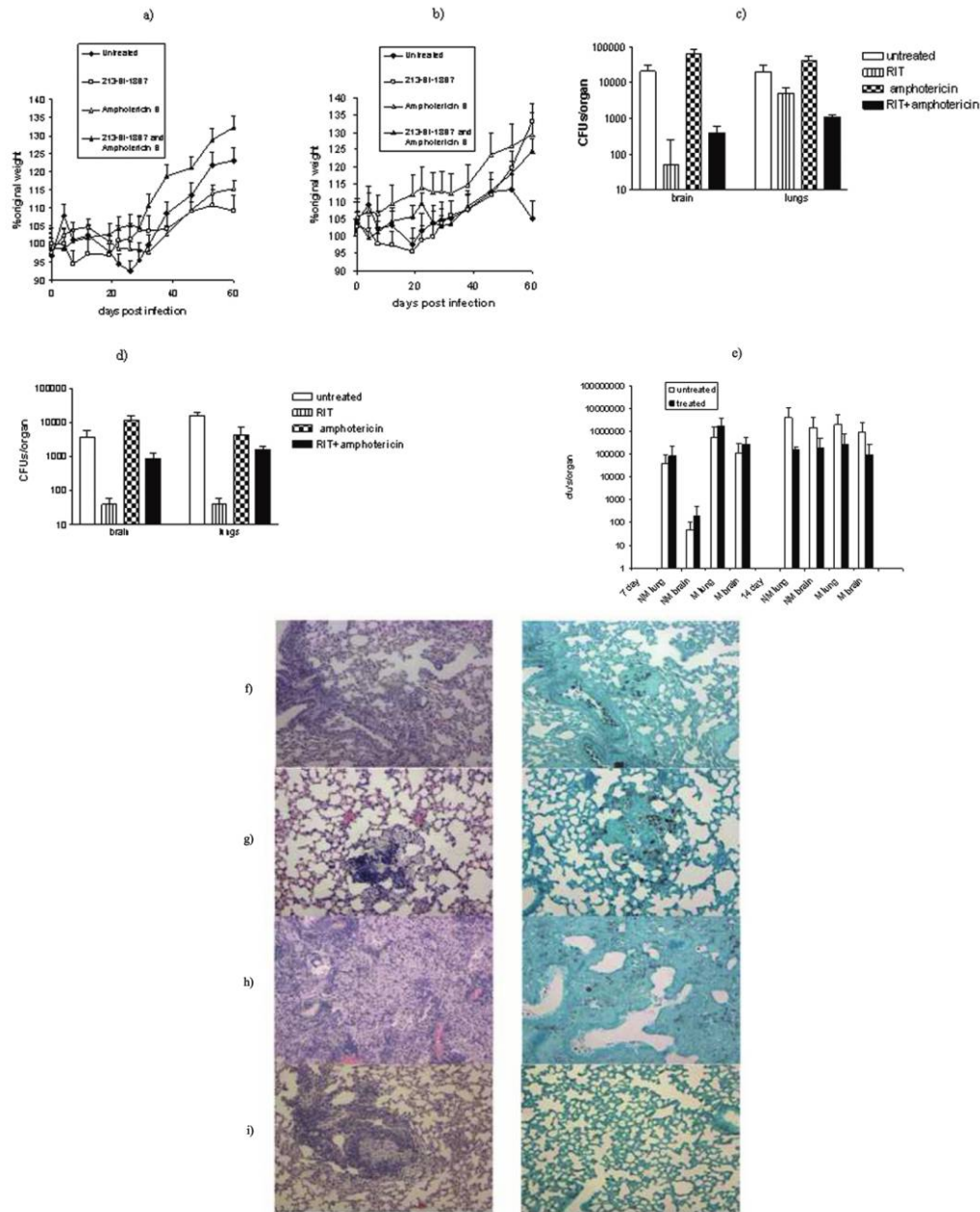
melanized *C. neoformans* group (Figure 2C) or the melanized *C. neoformans* group (Figure 2D) ( $P > .05$ ). RIT significantly decreased the fungal burden, compared with no treatment or amphotericin B treatment ( $P \ll .05$ ). In fact, RIT-treated mice in the nonmelanized *C. neoformans* group almost completely cleared fungus from the brain (lower limit of detection, 50 CFUs), whereas RIT-treated mice in the melanized *C. neoformans* group almost completely cleared the infection from both the brain and the lungs. The combined treatment was more efficient than amphotericin B alone for both brain and lungs ( $P < .05$ ) and more efficient than RIT alone in eliminating *C. neoformans* from the lungs ( $P < .05$ ) of mice in the nonmelanized *C. neoformans* group (Figure 2C). For mice in the melanized *C. neoformans* group, the combined treatment was better than amphotericin B alone for both the brain and the lungs ( $P < .05$ ) but worse than RIT alone ( $P < .05$ ) (Figure 2D).

Mice that received prolonged treatment with amphotericin B alone for 14 d showed no improvement in the number of CFUs in either the brain or the lungs on day 7 after treatment, in both the nonmelanized *C. neoformans* group and the me-

lanized *C. neoformans* group ( $P > .05$ ); on day 14 there was a trend toward a 1 log reduction in the number of CFUs in the brain and lungs of mice in both the nonmelanized *C. neoformans* group and the melanized *C. neoformans* group ( $P = .06$ ) (Figure 2E).

Histopathological analysis of lung and brain tissue demonstrated the focal nature of infection in all groups. Neutrophils and foamy macrophages infiltrated the lungs of mice that were untreated or treated with amphotericin B (Figures 2F and 2H). Interestingly, several mice that received RIT (Figure 2G) or combined treatment (Figure 2I) had some *C. neoformans* cells in the organs that were visualized by means of GMS staining but did not have CFUs in either the brain or the lungs, which indicates that fungal cells identified by GMS staining may have lost the ability to replicate.

**Discussion.** We compared the efficacy of RIT for infection with melanized or nonmelanized *C. neoformans* to that of the standard antifungal agent amphotericin B as a deoxycholate, as well as the efficacy of combination therapy. Melanin is a known virulence factor for *C. neoformans* [3], and melanization



**Figure 2.** Radioimmunotherapy (RIT) of mice infected with nonmelanized or melanized *Cryptococcus neoformans*. AJ/Cr mice were infected intravenously with  $3 \times 10^5$  *C. neoformans* cells and 24 h later either given 100  $\mu$ Ci of  $^{213}\text{Bi}$ -18B7 RIT; given amphotericin B at a dose of 1  $\mu$ g per gram of body weight on days 1, 2, and 3 after infection; given combined treatment; or left untreated. *a*, Body weights of mice infected with nonmelanized *C. neoformans*; *b*, Body weights of mice infected with melanized *C. neoformans*; *c*, Number of colony-forming units (CFUs) in the lungs and brains of treated and control mice infected with nonmelanized *C. neoformans*; *d*, Number of CFUs in the lungs and brains of treated and control mice infected with melanized *C. neoformans*. The detection limit of the method was 50 CFUs. No CFUs were detected in the brains and lungs of mice infected with melanized *C. neoformans* cells and treated with RIT; these measurements were given values of 40 CFUs per organ. *e*, Number of CFUs in the brain and lungs of mice infected with  $3 \times 10^5$  cells of melanized (M) or nonmelanized (NM) *C. neoformans* and treated with amphotericin B at a dose of 1  $\mu$ g per gram of body weight for 14 d. Mice were killed on days 7 and 14 after treatment. We performed histological analysis of consecutive sections of the lungs of the treated and control mice that were killed on day 60 after infection. *f*, Lung tissue sections from untreated mice; *g*, Lung tissue sections from RIT-treated mice; *h*, Lung tissue sections from mice treated with amphotericin B alone; *i*, Lung tissue sections from mice treated with a combination of RIT and amphotericin B. Tissues in the left panels are stained with hematoxylin and eosin; tissues in the right panels are stained with Gomori-Grocott methenamine silver. *C. neoformans* cells appear black on GMS-stained slides. Original magnification,  $\times 200$ .

protects *C. neoformans* from external  $\gamma$  radiation (as reviewed by Dadachova and Casadevall [10]). We observed that melanization of *C. neoformans* before infection did not increase the resistance of *C. neoformans* to RIT in vivo. Possible explanations for this may include the delicate balance existing in vivo between daughter cells that are not melanized right after budding [11] and the acquisition of melanin once the daughter cells establish themselves in organs containing melanin precursors, as well as the contribution of host immune defenses toward RIT efficacy [7], which is obviously absent in vitro. It has been demonstrated that mice mount an intense antibody response to fungal melanin that includes antibodies of immunoglobulin M and immunoglobulin G isotypes, which points to the stimulation of the immune system by melanin [12]. Given that antibodies to melanin can have a direct antifungal effect on cryptococcal cells, it is possible that such stimulation of the immune system contributes to the efficacy of RIT against melanized cells.

Our most important observation is that RIT was more effective in reducing the fungal burden in the lungs and brain than amphotericin B at a high dose of 1  $\mu\text{g/g}$ , with most RIT-treated mice almost completely clearing the infection. The inability of amphotericin B to reduce the fungal burden in the organs of partially complement-deficient AJ/Cr mice after 3 d of treatment was explained in the follow-up study, with a trend toward a reduction in the number of CFUs in the brain and lungs manifesting itself only on day 14 of treatment. These observations are in concert with those in literature, which show that even in intact robust mice (such as CD-1 or BALB/c mice), amphotericin B as deoxycholate was also able to produce only a 1–1.5 log reduction in the number of CFUs and all mice died around day 24 [13, 14]. Our observations are also in concert with the data from clinical studies showing that a short course of amphotericin B does not sterilize cerebrospinal fluid or blood and that the rate of sterilization correlates with survival [15]. Our results underline the advantages of RIT, which produces microbicidal effects in vivo after just 1 injection, compared with prolonged treatment with amphotericin B. When combined RIT and amphotericin B treatment was used, a complex picture emerged of dependence on the melanization status of the infection. Combination treatment was more effective than amphotericin B alone for both nonmelanized and melanized *C. neoformans* groups. For mice in the melanized *C. neoformans* group, the combination treatment was less effective than RIT, which could be due to the inflammation and renal toxicities associated with amphotericin B at this dose in mice. Interestingly, for mice in the nonmelanized *C. neoformans* group, the combination treatment did produce some synergy in reducing the number of CFUs in the lungs. It is possible to suggest that if RIT is administered much later during the course of treatment

with amphotericin B, then some synergistic effects might be observed.

It is noteworthy that the remaining fungal cells retain sensitivity to radiation and would be amenable to further RIT treatments [8]. In conclusion, RIT with  $\alpha$ -emitter-armed antibodies is more effective than amphotericin B against experimental systemic infections with both melanized and nonmelanized *C. neoformans*, which presents an attractive option for the development of clinical treatment.

## References

1. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **2009**; 23:525–530.
2. Ben-Ami R, Lewis RE, Kontoyiannis DP. Immunopharmacology of modern antifungals. *Clin Infect Dis* **2008**; 47:226–235.
3. Nosanchuk JD, Casadevall A. The contribution of melanin to microbial pathogenesis. *Cell Microbiol* **2003**; 5:203–223.
4. Sharkey RM, Goldenberg DM. Perspectives on cancer therapy with radiolabeled monoclonal antibodies. *J Nucl Med* **2005**; 46(suppl 1):115S–127S.
5. Dadachova E, Nakouzi A, Bryan RA, Casadevall A. Ionizing radiation delivered by specific antibody is therapeutic against a fungal infection. *Proc Natl Acad Sci U S A* **2003**; 100:10942–10947.
6. Dadachova E, Bryan RA, Frenkel A, et al. Evaluation of acute hematological and long-term pulmonary toxicity of radioimmunotherapy of *Cryptococcus neoformans* infection in murine models. *Antimicrob Agents Chemother* **2004**; 48:1004–1006.
7. Dadachova E, Bryan RA, Apostolidis C, et al. Interaction of radiolabeled antibodies with fungal cells and components of the immune system in vitro and during radioimmunotherapy for experimental fungal infection. *J Infect Dis* **2006**; 193:1427–1436.
8. Bryan RA, Jiang Z, Huang X et al. Radioimmunotherapy is effective against a high infection burden of *Cryptococcus neoformans* in mice and does not select for radiation-resistant phenotypes in cryptococcal cells. *Antimicrob Agents Chemother* **2009**; 53:1679–1682.
9. Dadachova E, Howell RW, Bryan RA, Frenkel A, Nosanchuk JD, Casadevall A. Susceptibility of human pathogens *Cryptococcus neoformans* and *Histoplasma capsulatum* to  $\gamma$ -radiation versus radioimmunotherapy with  $\alpha$ - and  $\beta$ -emitting radioisotopes. *J Nucl Med* **2004**; 45:313–320.
10. Dadachova E, Casadevall A. Ionizing radiation: how fungi cope, adapt and exploit with the help of melanin. *Curr Opin Microbiol* **2008**; 11: 525–531.
11. Nosanchuk JD, Casadevall A. Budding of melanized *Cryptococcus neoformans* in the presence or absence of L-DOPA. *Microbiology* **2003**; 149:1945–1951.
12. Nosanchuk JD, Rosas AL, Casadevall A. The antibody response to fungal melanin in mice. *J Immunol* **1998**; 160:6026–6031.
13. Clemons KV, Stevens DA. Comparison of Fungizone, Amphotec, AmBisome, and Abelcet for treatment of systemic murine cryptococcosis. *Antimicrob Agents Chemother* **1998**; 42:899–902.
14. Kakeya H, Miyazaki Y, Senda H, et al. Efficacy of SPK-843, a novel polyene antifungal, in a murine model of systemic cryptococcosis. *Antimicrob Agents Chemother* **2008**; 52:1871–1872.
15. Bicanic T, Muzaora C, Brouwer AE, et al. Independent association between rate of clearance of infection and clinical outcome of HIV-associated cryptococcal meningitis: analysis of a combined cohort of 262 patients. *Clin Infect Dis* **2009**; 49:702–709.