Radiofungicidal Effects of External Gamma Radiation and Antibody-Targeted Beta and Alpha Radiation on *Cryptococcus neoformans*[▽]

Ruth A. Bryan,[1] Xianchun Huang,[1] Alfred Morgenstern,[2] Frank Bruchertseifer,[2]
Arturo Casadevall,[3,4] and Ekaterina Dadachova[1,3]*

*Department of Nuclear Medicine[1] and European Commission,[2] Joint Research Centre, Institute for Transuranium Elements,
Karlsruhe, Germany, and Department of Microbiology and Immunology[3] and Department of Medicine,[4]
Albert Einstein College of Medicine, Bronx, New York*

We evaluated the clonogenic survival, membrane permeability, metabolic activity (XTT reduction), and apoptosis (FLICA binding) of *Cryptococcus neoformans* cells subjected to gamma rays from an external source, and beta and alpha particles delivered to fungal cells by capsule-specific antibody. We found that gamma, beta, and alpha radiation affected cells through different pathways.

Fungal cells such as *Cryptococcus neoformans* are extremely resistant to external gamma radiation (7), perhaps due to the extensive machinery that repairs UV-damaged DNA (11). In contrast, *C. neoformans* is highly susceptible to beta and alpha radiation delivered by monoclonal antibodies (MAbs) specific for the polysaccharide capsule of *C. neoformans* (7, 8, 14), implying that damage by particulate radiation is different from gamma radiation damage. The susceptibility to antibody-delivered particulate radiation is the basis for the radioimmunotherapy (RIT) of cryptococcal infection (7, 8). RIT of *C. neoformans*-infected mice with alpha-emitter ^{213}Bi- or beta-emitter ^{188}Re-labeled *C. neoformans* capsule-specific MAbs produced effects on both the fungal cells and the host immune system (6). Given that RIT of infectious diseases is a relatively young field, the mechanisms by which RIT is effective in murine cryptococcosis (7, 8) are uncertain. Even in oncology, where the anti-neoplastic effects of RIT have been investigated for more than 25 years, the mechanisms are still debated. The major radiobiological mechanisms of cancer RIT are considered to be "direct-hit" and "crossfire" effects, both of which can promote apoptosis and cell cycle redistribution (12). Here we investigated the radiofungicidal effects of both external radiation and radiolabeled MAbs on *C. neoformans* cells by evaluating the influence of radiofungicidal doses on cell membrane permeability, the induction of apoptosis, and cellular metabolism.

(These results were presented in part at the 106th General Meeting of the American Society for Microbiology, Orlando, FL, 21 to 25 May 2006, abstr. F-023.)

Gamma radiation induced membrane permeability in a percentage of *C. neoformans* cells, while radiolabeled antibodies did not. We compared the damage to *C. neoformans* cells caused by gamma radiation to that caused by treatment with ^{213}Bi- or ^{188}Re-labeled 18B7, a mouse MAb (immunoglobulin G1) recognizing the polysaccharide capsule of *C. neoformans*

(5). *C. neoformans* cells (strain 24067, obtained from the American Type Culture Collection, Manassas, VA) were grown as described previously (6). Cells were irradiated with ^{137}Cs at 14 Gy/min for up to 600 Gy, followed by plating for CFU, to measure the cells' clonogenic survival. During a 7-day observation of the plates with radiation-treated *C. neoformans* cells plated for clonogenic survival, the colonies appeared simultaneously on day 3, with no new colonies appearing during the rest of the 7-day observation period, a finding indicating no of reactivation of the dormant cells. Since in vitro there is no suppression of the cells by the host's immune system, it is unlikely that radiation treatment left behind any cells capable of reactivation. For RIT experiments MAb 18B7 was radiolabeled with ^{213}Bi and ^{188}Re as described earlier (6, 8). For that purpose, ^{213}Bi, which emits primarily alpha as well as some beta particles, was eluted from a ^{225}Ac generator from the Institute for Transuranium Elements, Karlsruhe, Germany (1); ^{188}Re, a beta emitter, was eluted from a ^{188}W/^{188}Re generator (Oak Ridge National Laboratories, Oak Ridge, TN). *C. neoformans* cells were incubated with radiolabeled antibodies for 1 h at 37°C, the unbound antibodies were removed, and the cells were washed with phosphate-buffered saline. For ^{188}Re-18B7 MAb the cells were incubated with shaking in phosphate-buffered saline at 4°C for 2 days to allow ^{188}Re with its physical half-life of 16.9 h to deliver its radiation dose; for ^{213}Bi-18B7 MAb 3 h of incubation at room temperature was sufficient, since ^{213}Bi has a short physical half-life of 46 min. All three forms of radiation decreased the clonogenic survival, with the doses that caused losses of 80 to 100% being 250 Gy for gamma radiation, 1 Gy for ^{188}Re-18B7, and 0.5 Gy for ^{213}Bi-18B7 (Fig. 1). The doses for radiolabeled MAbs were calculated by using an algorithm previously developed for RIT of fungal cells (7). We observed no killing of *C. neoformans* cells when gamma radiation was delivered to *C. neoformans* cells by 18B7 MAb radiolabeled with a pure gamma emitter 99m-technetium (^{99m}Tc; 140 keV gamma emission with 89% abundance) at the dose range of 0.7 to 45 μCi/10^5 cells (not shown).

One marker of cell death is an increased membrane permeability to the dye propidium iodide (PI), which is excluded

* Corresponding author. Mailing address: Albert Einstein College of Medicine, Department of Nuclear Medicine, 1695A Eastchester Rd., Bronx, NY 10461. Phone: (718) 405-8485. Fax: (718) 405-8457. E-mail: edadacho@aecom.yu.edu.
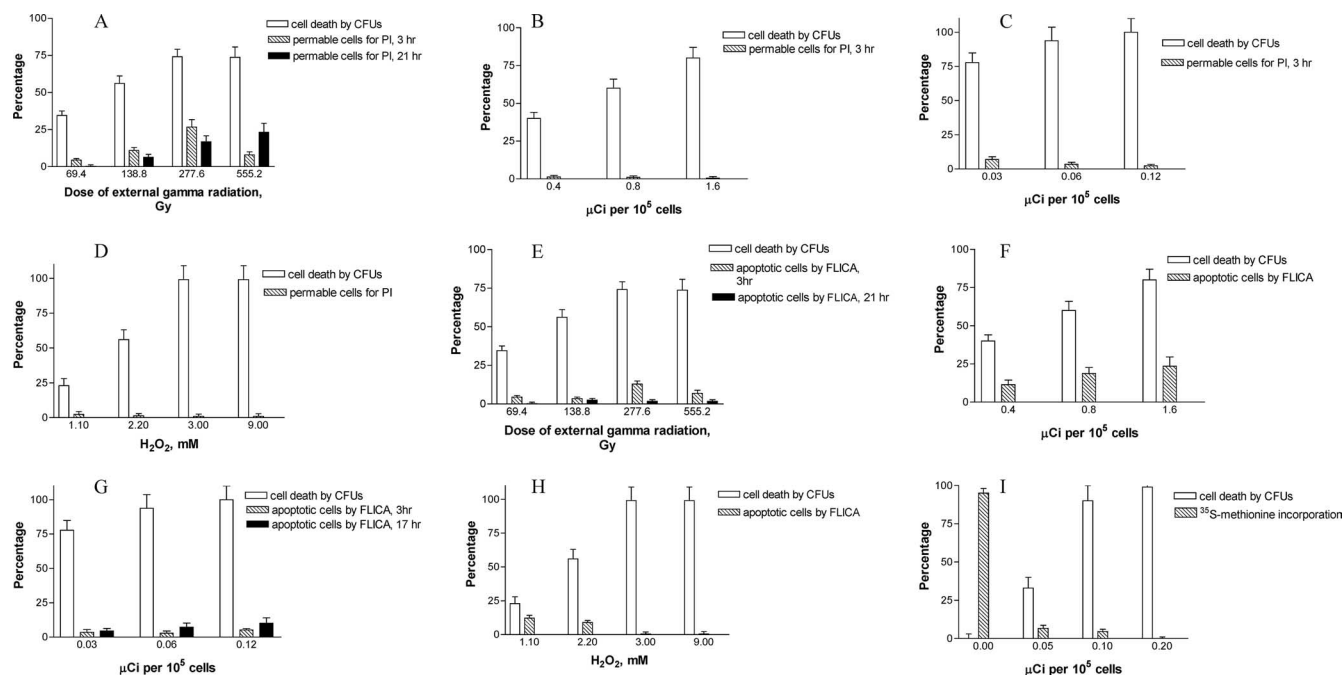
FIG. 1. Comparison of the percentage of nonviable *C. neoformans* cells by CFU, PI permeability, and apoptosis levels by FLICA for radiation-treated cells. Panels A to D show CFU and PI permeability. (A) External gamma radiation; (B) [188]Re-18B7; (C) [213]Bi-18B7; (D) H_2O_2 controls. Panels E to H show CFU and apoptosis levels by FLICA. (E) External gamma radiation; (F) [188]Re-18B7; (G) [213]Bi-18B7; (H) H_2O_2 controls. (I) CFU and [^{35}S]methionine incorporation by [213]Bi-18B7-treated *C. neoformans* cells.

from cells with intact membranes. Internalized PI binds to nucleic acids and undergoes a large increase in fluorescence (9). PI staining correlates well with the loss of CFU in a variety of organisms, including *C. neoformans* treated with antifungal agents (2). More than 95% of heat-killed *C. neoformans* cells were PI positive and served as positive control, while untreated *C. neoformans* cells were negative controls (not shown). Fluorescence was measured on the FL-2 channel of a FACScan (Becton Dickinson), and the data were analyzed by using CellQuest software. Cells stained with PI immediately or 1 h after gamma irradiation showed no uptake of PI (not shown). The permeability increased with time between 1 and 3 h after gamma irradiation, indicating that it was probably secondary to cell death and not a cause of death. It seems likely that the cells in this 20% of the population are metabolically "dead" and unable to maintain membrane integrity. Cells stained 3 h after irradiation showed dose-dependent PI staining up to ~300 Gy (25% PI positive), with a decrease to 10% PI positive at the highest dose (Fig. 1A). This observation indicates again that membrane damage is not the primary lethal event, since 80% of the cells had lost clonogenic ability at these doses. The decrease in PI-positive cells at the highest dose may be due to protective effects from the shed capsule (4). Damage to the membrane as measured by PI permeability did not increase by 21 h. Treatment of *C. neoformans* with [188]Re-18B7 did not make the cells PI permeable (Fig. 1B). Treatment with [213]Bi-18B7 MAb led to ca. 7% of the cells becoming PI permeable, at a dose that caused 80% loss of CFU (Fig. 1C). Higher doses of [213]Bi-18B7 MAb actually decreased the permeability. For comparison, the *C. neoformans* cells were treated with H_2O_2

for 3 h, which induced high CFU losses but did not make the cells permeable to PI (Fig. 1D).

All radiation types initiated the process of apoptosis in *C. neoformans* cells. Fungal cells undergo apoptosis or programmed cell death (13). We investigated whether radiation increased levels of fungal caspase, as measured by FLICA (for fluorochrome-labeled inhibitor of caspase) binding, a membrane-permeable substrate that binds to caspases induced during early apoptosis. Earlier, we validated this technique for use with *C. neoformans* by comparing the FLICA results to those obtained using an APO-BrdU TUNEL apoptosis detection kit (6). Cell fluorescence was measured on the FL-1 channel of the FACScan. Gamma-irradiated cells were about 10% FLICA positive at 3 h (Fig. 1E), whereas 20 and 5% of *C. neoformans* cells exposed to [188]Re-18B7 or [213]Bi-18B7 MAbs, respectively, became FLICA positive (Fig. 1F and G). The number of FLICA-positive [213]Bi-18B7 MAb-treated cells staining was higher at 17 h than at 3 h, indicating an ongoing process of apoptosis induction. Apoptosis is a dynamic process, and cells pass through several stages, not staying at any one stage for a long time. The decrease seen at 21 h for the gamma-radiation-treated cells may indicate that at 21 h the cells have finished the stage of apoptosis during which the caspases are available to bind the fluorescent inhibitors. This is in contrast to the increase with time observed for [213]Bi-18B7 MAb-treated cells and may reflect a difference in pathways of cell death induced by the different forms of radioactivity. The levels of induction were similar to those after treatment with 1 to 3 mM H_2O_2, a standard positive control for apoptosis induction (Fig. 1H).
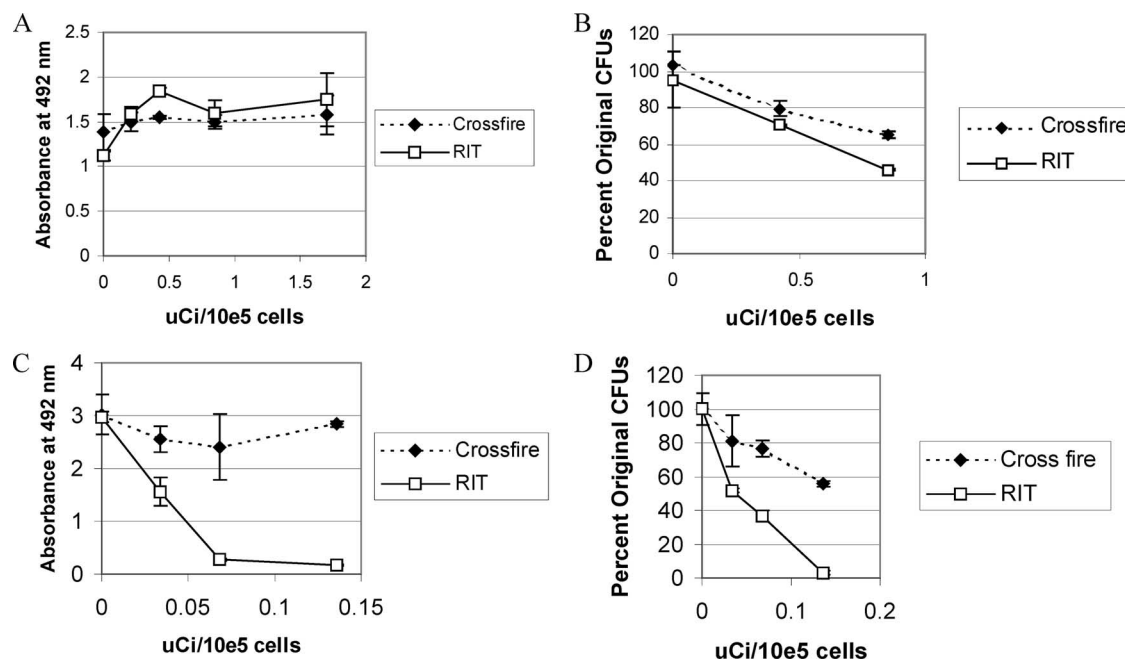
FIG. 2. Comparison of the percentage of nonviable *C. neoformans* cells by CFU and metabolic activity by XTT assay for treatment with radiolabeled MAbs and its "crossfire" component. (A) XTT results for [188]Re-18B7; (B) CFU for [188]Re-18B7; (C) XTT results for [213]Bi-18B7; (D) CFU for [213]Bi-18B7.

Untreated cells had a 1 to 2% FLICA-positive population (not shown).

XTT assay detected the loss in metabolic activity only for cells "directly" hit with alpha-radiation. The cells ability to reduce the dye 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) depends on the availability of NADPH at the cell surface. XTT reduction is considered to be a measure of metabolic activity (3) and has been used to measure the antifungal activity of the drugs (10). Previously, we showed that gamma radiation did not affect XTT activity of *C. neoformans* cells even at doses as high as 830 Gy, whereas [188]Re-18B7 treatment caused a detectable decrease in XTT activity only at doses 10 times higher than those required for a 50% loss of CFU (14). In contrast, the XTT activity of *C. neoformans* cells treated with [213]Bi-18B7 MAb decreased to the same extent as their CFU (14). These results indicated that the damage required for the loss of XTT activity is distinct from the damage required for loss of the ability to replicate.

As another measure of metabolic activity, we used a [35S]methionine uptake assay to evaluate the metabolic activity of the cells treated with [213]Bi-18B7. The results showed that [35S]methionine incorporation decreased in parallel with CFU, starting from 18 h of incubation with the [213]Bi-18B7-treated cells (Fig. 1I).

In the present study we investigated the mechanism of this phenomenon by comparing the ability of RIT to the ability of its "crossfire" component to interfere with metabolism of *C. neoformans* as measured by an XTT assay. As assumed in the field of RIT, the cells could be killed by antibody-delivered radiation via a "direct-hit" (killing of a cell by radiation emanating from a radiolabeled molecule bound to this cell) or

"crossfire" (killing of a cell by radiation emanating from a radiolabeled molecule bound to an adjacent or distant cell) effects or a combination of both. A "crossfire" effect is essential for the success of cancer RIT (12). Cells that would serve as sources of "crossfire" radiation were prepared by incubating heat-killed *C. neoformans* cells with radiolabeled MAbs for 1 h at 37°C. The unbound antibodies were removed by centrifugation, and the dead cells with bound radiolabeled MAb were mixed with live *C. neoformans* cells to deliver the "crossfire" radiation to live cells. The mixture was incubated for 1 h at 37°C and assayed as described above for CFU and for XTT activity as described previously (14). Both RIT with [188]Re-18B7 MAb and its "crossfire" component, while equally decreasing CFU (Fig. 2B), did not change the levels of metabolism as compared to untreated cells (Fig. 2A). [213]Bi-18B7 RIT, on the other hand, produced a large decrease in XTT activity (Fig. 2C) that was proportional to the decrease in CFU (Fig. 2D). Interestingly, "crossfire" from [213]Bi-18B7 was unable to cause a decrease in XTT activity (Fig. 2C), indicating that there is a fundamental difference in the fungicidal mechanisms of "direct" RIT and its "crossfire" component for this radionuclide. The "crossfire" effect on CFU may be caused by the beta radiation component of [213]Bi decay, which has a longer range in tissue and is much less damaging than its alpha decay component.

In conclusion, we evaluated the clonogenic survival, membrane permeability, metabolic activity (XTT reduction), and apoptosis (FLICA binding) of *C. neoformans* cells subjected to gamma rays from an external source and beta and alpha particles delivered to fungal cells by capsule-specific antibody. We found that gamma, beta, and alpha radiation affected cells via different pathways. Gamma radiation had more effect on the

cell membrane than ^{213}Bi-18B7 or ^{188}Re-18B7. All forms of radiation stimulated apoptosis, with gamma radiation and ^{188}Re-18B7 MAb having a more pronounced effect than ^{213}Bi-18B7 MAb. ^{213}Bi-18B7 MAb delivered "directly" decreased the metabolic activity of fungal cells, while the other forms of radiation did not. Clonogenic survival proved to be the most practical measure for assessing RIT efficacy by virtue of reflecting a combination of multiple mechanisms leading to fungal cell death. Cells that are alive after RIT treatment, but not replicating, may or may not contribute to the disease. Knowledge of the radiobiological mechanisms of RIT will allow creation of more effective protocols for RIT of opportunistic fungal infections.

REFERENCES

1. **Apostolidis, C., R. Molinet, G. Rasmussen, and A. Morgenstern.** 2005. Production of Ac-225 from Th-229 for targeted alpha therapy. Anal. Chem. **77:**6288–6291.
2. **Benincasa, M., M. Scocchi, S. Pacor, A. Tossi, D. Nobili, G. Basaglia, M. Busetti, and R. Gennaro.** 2006. Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts. J. Antimicrob. Chemother. **58:**950–959.
3. **Berridge, M. V., P. M. Herst, and A. S. Tan.** 2005. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol. Annu. Rev. **11:**127–152.
4. **Bryan, R. A., O. Zaragoza, T. Zhang, G. Ortiz, A. Casadevall, and E. Dadachova.** 2005. Radiological studies reveal radial differences in the architecture of the polysaccharide capsule of *Cryptococcus neoformans.* Eukaryot. Cell **4:**465–475.
5. **Casadevall, A., J. Mukherjee, S. J. Devi, R. Schneerson, J. B. Robbins, and M. D. Scharff.** 1992. Antibodies elicited by a *Cryptococcus neoformans*-tetanus toxoid conjugate vaccine have the same specificity as those elicited in infection. J. Infect. Dis. **165:**1086–1093.
6. **Dadachova, E., R. A. Bryan, C. Apostolidis, A. Morgenstern, T. Zhang, T. Moadel, M. Torres, X. Huang, E. Revskaya, and A. Casadevall.** 2006. 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. **193:**1427–1436.
7. **Dadachova, E., R. W. Howell, R. A. Bryan, A. Frenkel, J. D. Nosanchuk, and A. Casadevall.** 2004. Susceptibility of the human pathogenic fungi *Cryptococcus neoformans* and *Histoplasma capsulatum* to gamma-radiation versus radioimmunotherapy with alpha- and beta-emitting radioisotopes. J. Nuclear Med. **45:**313–320.
8. **Dadachova, E., A. Nakouzi, R. A. Bryan, and A. Casadevall.** 2003. Ionizing radiation delivered by specific antibody in therapeutic against a fungal infection. Proc. Natl. Acad. Sci. USA **100:**10942–10947.
9. **Deere, D., J. Shen, G. Vesey, P. Bell, P. Bissinger, and D. Veal.** 1998. Flow cytometry and cell sorting for yeast viability assessment and cell selection. Yeast **14:**147–160.
10. **Hawser, S. P., H. Norris, C. J. Jessup, and M. A. Ghannoum.** 1998. Comparison of a 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) colorimetric method with the standardized National Committee for Clinical Laboratory Standards method of testing clinical yeast isolates for susceptibility to antifungal agents. J. Clin. Microbiol. **36:**1450–1452.
11. **Krogh, B. O., and L. Symington.** 2004. Recombination proteins in yeast. Annu. Rev. Genet. **38:**233–271.
12. **Macklis, R. M.** 2004. How and why does radioimmunotherapy works? Int. J. Radiat. Oncol. Biol. Phys. **59:**1269–1271.
13. **Madeo, F., E. Herker, S. Wissing, H. Jungwirth, T. Eisenberg, and K. U. Frohlich.** 2004. Apoptosis in yeast. Curr. Opin. Microbiol. **7:**655–660.
14. **Martinez, L. R., R. A. Bryan, C. Apostolidis, A. Morgenstern, E. Dadachova, and A. Casadevall.** 2006. Antibody-guided alpha-radiation effectively damages fungal biofilms. Antimicrob. Agents Chemother. **50:**2132–2136.