Susceptibility of the Human Pathogenic Fungi *Cryptococcus neoformans* and *Histoplasma capsulatum* to γ-Radiation Versus Radioimmunotherapy with α- and β-Emitting Radioisotopes

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Fungal diseases are difficult to treat in immunosuppressed patients and, consequently, new approaches to therapy are urgently needed. One novel strategy is to use radioimmunotherapy (RIT) with fungal-binding monoclonal antibodies (mAbs) labeled with radionuclides. However, many fungi manifest extreme resistance to γ-radiation, such that the doses of several thousand gray are required for 90% cell killing, whereas for mammalian cells the lethal dose is only a few gray. We compared the susceptibility of human pathogenic fungi *Cryptococcus neoformans* (CN) and *Histoplasma capsulatum* (HC) to external γ-radiation and to the organism-specific mAbs 18B7 and 9C7, respectively, conjugated to ^{213}Bi and ^{188}Re radionuclides. **Methods:** CN and HC cells were irradiated with up to 8,000 Gy (^{137}Cs source, 30 Gy/min). RIT of CN with ^{213}Bi- and ^{188}Re-labeled specific mAb and of HC with ^{188}Re-labeled specific mAb used 0–1.2 MBq per 10^5 microbial cells. After irradiation or RIT, the cells were plated for colony-forming units (CFUs). Cellular dosimetry calculations were performed, and the pathway of cell death after irradiation was evaluated by flow cytometry. **Results:** Both CN and HC proved to be extremely resistant to γ-radiation such that significant killing was observed only for doses of >4,000 Gy. In contrast, these cells were much more susceptible to killing by radiation delivered with a specific mAb, such that a 2-logarithm reduction in colony numbers was achieved by incubating them with ^{213}Bi- and ^{188}Re-labeled mAb 18B7 or with ^{188}Re-9C7 mAb. Dosimetry calculations showed that RIT was ~1,000-fold more efficient in killing CN and ~100-fold more efficient in killing HC than γ-radiation. Both γ-radiation and RIT caused cell death via an apoptotic-like pathway with a higher percentage of apoptosis observed in RIT-treated cells. **Conclusion:** Conjugating a radioactive isotope to a fungal-specific antibody converted an immunoglobulin with no antifungal activity into a microbicidal molecule. RIT of fungal cells using specific antibodies labeled with α- and β-emitting radioisotopes was significantly more efficient in killing CN and HC than γ-radiation when based on the mean absorbed dose to the cell. These results strongly support the concept of using RIT as an antimicrobial modality.

Key Words: radioimmunotherapy; infection; dosimetry; pathogenic fungi

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Many fungi manifest extreme radioresistance to external γ-radiation relative to other microorganisms and mammalian cells (*1–5*). For example, a dose of several thousand gray is required to achieve 90% cell killing of fungal cells, whereas the lethal dose for mammalian cells is only a few gray. The mechanisms responsible for these differences are not well understood but could involve more efficient mechanisms of DNA repair by fungi when the damage is caused by γ-rays. A dramatic example of the radioresistance of fungi is provided by reports of numerous melanotic fungal species colonizing the walls of the damaged nuclear reactor at Chernobyl in extremely high radiation fields (*6*).

We have recently shown that the human pathogenic fungus *Cryptococcus neoformans* (CN) is susceptible to killing by radioimmunotherapy (RIT) in vitro with an organism-specific monoclonal antibody (mAb) radiolabeled with the α-emitter ^{213}Bi or the β-emitter ^{188}Re (*7*). For the purposes of this study, the term "RIT" is used to refer to the microbicidal effects of radiolabeled specific mAb on fungal cells in vitro. This process reflects an enhanced microbicidal activity resulting from the delivery of a radionuclide to the

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fungal cell by a specific antibody that binds to the microbe. Furthermore, we established that this modality was effective therapeutically in a murine model of experimental cryptococcosis (*7*). This result was encouraging for developing a new therapy for cryptococcosis since this fungus is a major cause of chronic and often lethal illness in immunocompromised individuals (*8*). Our protocol used radiolabeled mAb with activities of 1.85–7.4 MBq (50–200 μCi) per mouse, which were fungicidal in vivo. By using an antibody to deliver a radionuclide to the fungal cell surface we observed microbicidal effects with radiation doses that were several orders of magnitude lower than expected from assumptions based on studies using external γ-radiation sources. Consequently, by attaching a radionuclide to an immunoglobulin we converted an antibody with no inherent antifungal activity into a microbicidal molecule.

The apparent efficacy of RIT as an antifungal therapeutic modality may appear to be in conflict with older reports that fungi are highly resistant to radiation. Therefore, we have revisited the question of fungal susceptibility to radiation by studying the microbicidal properties of various types of radiation using 2 medically relevant fungal species CN and *Histoplasma capsulatum* (HC). CN and HC are the most common causes of fungal meningitis and pneumonia, respectively (*8*,*9*). Antibodies for HC have recently been described (*10*) and were available for use in RIT. In this study we have analyzed the susceptibility of CN and HC to γ-radiation versus RIT with α- and β-emitting radioisotopes, performed extensive cellular dosimetry calculations, and investigated the pathway of cell death after irradiation by flow cytometry.

MATERIALS AND METHODS

CN and HC

American Type Culture Collection strains CN 24067 (serotype D, encapsulated), acapsular mutant CAP67 (parent strain B3501), and HC (CIB 1980; a gift from Dr. A. Restrepo, Medellin, Colombia) were used in all experiments. CN was grown in Sabouraud dextrose broth (Difco Laboratories) for 24 h at 30°C with constant shaking at 150 rpm. HC was grown in defined media containing 29.4 mmol/L KH_2PO_4, 10 mmol/L $MgSO_4 \times 7H_2O$, 13 mmol/L glycine, 15 mmol/L D-glucose, and 3 μmol/L thiamine with shaking at 37°C. Organisms were washed 3 times with phosphate-buffered saline (PBS), pH 7.2, before use.

Antibodies

mAb 18B7 (IgG1) binds to CN capsular polysaccharide (*11*). mAb 9C7 (IgM) binds to a 17-kDa protein antigen on the surface of the HC cell wall (*10*). mAb MOPC21 (ICN Biomedicals) was used as an irrelevant isotype-matched control for 18B7, and mAb UNLB (clone 11E10; Southern Biotechnology Associates) was used for 9C7.

Radioisotopes and Quantification of Radioactivity

^{188}Re in the form of Na perrhenate $Na^{188}ReO_4$ was eluted from a ^{188}W/^{188}Re generator (Oak Ridge National Laboratory [ORNL]). ^{225}Ac for construction of a ^{225}Ac/^{213}Bi generator was acquired from ORNL. The ^{225}Ac/^{213}Bi generator was constructed using an MP-50 cation-exchange resin column and ^{213}Bi was eluted with 0.15 mol/L hydroiodic acid in the form of $^{213}BiI_3$ as described (*12*).

The radioactive samples were counted in a dose calibrator when their activity was >185 kBq (>5 μCi) and counted in a γ-counter with or without appropriate dilution when their activity was <185 kBq (<5 μCi). A γ-counter (Wallac) with an open window was used to count the ^{188}Re and ^{213}Bi samples. Samples containing known activities were counted to obtain experimentally determined conversion factors of 23 cpm/Bq (840,000 cpm/μCi) for ^{188}Re and 41 cpm/Bq (1,500,000 cpm/μCi) for ^{213}Bi.

Susceptibility of CN and HC to External γ-Radiation

Approximately 10^5 CN or HC cells were placed in microcentrifuge tubes in 0.5 mL PBS and irradiated at room temperature in a cell irradiator equipped with a ^{137}Cs source at a dose rate of 30 Gy/min. The cells were exposed to doses of up to 8,000 Gy (800 krad). To determine if unlabeled antibody might contribute to killing of fungal cells, in several experiments CN or HC cells were irradiated with 4,000 Gy (0–400 krad) in the presence of 10 μg 18B7 or 9C7 mAb, respectively. The potential protective effect of the polysaccharide capsule surrounding CN cells was investigated by irradiating acapsular CN strain CAP67. After radiation exposure, 10^3 cells were removed from each tube, diluted with PBS, and plated to determine viability as measured by colony-forming units (CFUs). Experiments were performed in duplicate.

Radiolabeling of Antibodies with ^{188}Re and ^{213}Bi

Our previously described procedure (*7*) was used. Briefly, mAbs were labeled directly with ^{188}Re via reduction of antibody disulfide bonds by incubating the antibody with a 75-fold molar excess of dithiothreitol for 40 min at 37°C followed by centrifugal purification on Centricon-30 or Centricon-50 microconcentrators with 0.15 mol/L NH_4OAc, pH 6.5. Simultaneously, 110–370 MBq (3–10 mCi) $^{188}ReO_4^-$ in saline were reduced with $SnC1_2$ by incubation in the presence of Na gluconate, combined with purified reduced antibodies and kept at 37°C for 60 min. Radioactivity not bound to the antibody was removed by centrifugal purification on Centricon microconcentrators.

For radiolabeling with ^{213}Bi, mAbs were conjugated to the bifunctional chelator *N*-[2-amino-3-(*p*-isothiocyanatophenyl)propy1]-trans-cyclohexane-1,2-diamine-*N*,*N*′,*N*″,*N*‴,*N*⁗-pentaacetic acid (CHXA″). CHXA″-conjugated mAbs were radiolabeled with ^{213}Bi by incubating them for 5 min with $^{213}BiI_3$ at room temperature. If required, the radiolabeled antibodies were purified by size-exclusion high-performance liquid chromatography (TSK-Gel G3000SW; TosoHaas).

In Vitro RIT of CN and HC with Radiolabeled Antibodies

The RIT of HC was performed only with ^{188}Re-labeled antibodies as 9C7 mAb proved to be not amenable to conjugation with CHXA″ for further radiolabeling with ^{213}Bi. ^{188}Re- or ^{213}Bi-radiolabeled mAb 18B7 and ^{188}Re-radiolabeled 9C7 were used to determine the susceptibility of CN and HC to RIT, respectively, in vitro. mAbs MOPC21 and UNLB were radiolabeled in the same manner and served as isotype-matching controls for 18B7 and 9C7, respectively. The cells were incubated at 37°C for 30 min in PBS containing 10 μg ^{188}Re-mAb (1.2 MBq [0–32 μCi]) or ^{213}Bi-mAb (0.12 MBq [0–3.2 μCi]) to allow time for the radiolabeled antibodies to bind to the cell surfaces. For the control, CN or HC cells were incubated with 10 μg prereduced or CHXA″-conjugated unlabeled mAbs. To measure the kinetics of antibody binding to the cells, aliquots of cell suspension were removed at 0,

10, 20, and 30 min, centrifuged to separate the cell pellet from the supernatant, and the cell pellet was counted in a γ-counter. After a 30-min incubation with the antibodies, the cells were washed free of extracellular activity and maintained in suspension on a rocker at 4°C in PBS for a period of either 1 or 48 h for ^{213}Bi-mAbs or ^{188}Re-mAbs, respectively. The cells were not dividing during the incubation and maintenance periods because they were maintained in PBS, which lacks nutrients. To ascertain that the radioactivity remained bound to the cells at the end of the 1- or 48-h incubation period, aliquots of cell suspension were removed, counted in the γ-counter, centrifuged to separate the cell pellet from the supernatant, and washed with PBS, and the cell pellet was counted in a γ-counter. At the end of incubation, 10^3 cells were plated for CFUs and the colonies were counted after 48 h at room temperature.

Cellular Dosimetry

Following the general formalism for cellular dosimetry given by Equation 7 in (*13*), the mean absorbed dose to the cell from cellular radioactivity is given by:

$$D_c = \tilde{A} \sum_j b_j S_j(C \leftarrow CS), \quad \text{Eq. 1}$$

where $\tilde{A}$ is the cellular cumulated activity, b_j is the branching ratio of the jth radionuclide in the decay series, and $S_j(C\leftarrow CS)$ is the cellular S value (absorbed dose to the cell per unit cumulated activity) for the jth radionuclide localized on the cell surface (CS) of the cell (C). The cumulated activity $\tilde{A}$ can be written as:

$$\tilde{A} = \tilde{A}_I + \tilde{A}_M + \tilde{A}_{CF}, \quad \text{Eq. 2}$$

where $\tilde{A}_I$, $\tilde{A}_M$, and $\tilde{A}_{CF}$ are the cellular cumulated activities during the periods of incubation for cellular uptake of radioactivity, maintenance at 4°C, and colony formation, respectively.

Cumulated Activity During Incubation Period at 37°C. According to Makrigiorgos et al. (*14*), when the cellular uptake is linear in time and there is no biologic elimination of the radioactivity, the cellular radioactivity at time *t* of the incubation period is given by:

$$A_I(t) = kA_o te^{-\lambda t}, \quad \text{Eq. 3}$$

where k is a constant of proportionality determined experimentally, A_o is the initial activity added to the tube containing the cells, and λ is the physical decay constant for the radionuclide ($\lambda = 0.693/T_p$, where T_p is the physical half-life). Makrigiorgos et al. (*14*) have also shown that the cumulated activity at time $t = t_I$ is given by:

$$\tilde{A}_I(t = t_I) = \frac{k}{\lambda^2} A_o\{1 - (1 + \lambda t_I)e^{-\lambda t_I}\}. \quad \text{Eq. 4}$$

Cumulated Activity During Maintenance Period at 4°C. The activity present in the cell at the beginning of the maintenance period is given by:

$$A_M(t_I) = kA_o t_I e^{-\lambda t_I}. \quad \text{Eq. 5}$$

Assuming no biologic elimination of the radioactivity from the cells, the cellular activity as a function of time *t* during the maintenance period is then:

$$A_M(t) = kA_o t_I e^{-\lambda t_I} e^{-\lambda t}. \quad \text{Eq. 6}$$

The cumulated activity during the maintenance period t_M is then given by:

$$\tilde{A}_M(t = t_M) = kA_o t_I e^{-\lambda t_I} \int_o^{t_M} e^{-\lambda t} dt = \frac{k}{\lambda} A_o t_I e^{-\lambda t_I}(1 - e^{-\lambda t_M}). \quad \text{Eq. 7}$$

Cumulated Activity During Colony-Forming Period at Room Temperature. Finally, assuming that the activity is eliminated from the cells by both biologic processes of an exponential nature and physical decay, the activity present in the cell at time *t* during the colony-forming period is given by:

$$A_{CF}(t) = kA_o t_I e^{-\lambda t_I} e^{-\lambda t_M} e^{-\lambda_e t}. \quad \text{Eq. 8}$$

The quantity λ_e is the effective decay constant, where $\lambda_e = \lambda + \lambda_b$, and λ_b is the biologic disappearance constant (*15*). The cumulated activity during the colony-forming period t_{CF} is therefore given by:

$$\tilde{A}_{CF}(t = t_{CF}) = kA_o t_I e^{-\lambda t_I} e^{-\lambda t_M} \int_o^{t_{CF}} e^{-\lambda_e t}\, dt$$

$$= \frac{k}{\lambda_e} A_o t_I e^{-\lambda t_I} e^{-\lambda t_M}(1 - e^{-\lambda_e t_{CF}}). \quad \text{Eq. 9}$$

Cellular S Values and Physical Decay Constants. The cellular S values are obtained from the tabulations in the Society of Nuclear Medicine's *MIRD Cellular S Values* monograph (*13*). Since the antibodies remain on the cell surfaces, only $S(C\leftarrow CS)$ is required. The radii of the CN and HC cells are approximately 10 and 5 μm, respectively. The corresponding S values for ^{188}Re are 6.82×10^{-5} and 3.34×10^{-4} Gy $Bq^{-1}s^{-1}$ for CN and HC, respectively. As expected, the cellular S values are quite sensitive to cell size. Deciding which S values are required for ^{213}Bi dosimetry is more complex due to its decay to various daughter radionuclides (Fig. 1). The violence of the ^{213}Bi α-decay can be assumed to disrupt the radionuclide-antibody bond with consequent departure of the daughter ^{209}Tl from the cell surface before it has a chance to decay. The same is assumed for ^{209}Pb, the daughter of ^{213}Po. Therefore, only S values for ^{213}Bi and ^{213}Po are required. For ^{213}Bi localized on the surface of cells having radii of 10 and 5 μm, $S(C\leftarrow CS)$ is 4.14×10^{-4} and 1.64×10^{-3} Gy $Bq^{-1}s^{-1}$, respectively. No S values are tabulated in (*13*) for ^{213}Po, which emits an 8.375-MeV α-particle. Interpolation of the cellular S values for α-particles given in Appendix III of *MIRD Cellular S Values* (*13*) gives $S(C\leftarrow CS) = 1.21 \times 10^{-2}$ and 4.74×10^{-2} Gy $Bq^{-1}s^{-1}$ for ^{213}Po on the surface of cells with radii of 10 and 5 μm, respectively. The physical decay constant for a radionuclide is given by $\lambda = 0.693/T_p$. Therefore, $\lambda(^{188}\text{Re}) = 6.80 \times 10^{-4}$ min^{-1} and $\lambda(^{213}\text{Bi}) = 1.52 \times 10^{-2}$ min^{-1}.

Flow Cytometry

Flow cytometry can be used to identify cells undergoing apoptosis. After γ-irradiation or RIT, the cells were permeabilized and fixed with 70% ethanol, cellular RNA was destroyed by treatment with ribonuclease A at 50°C for 1 h, the DNA was stained with propidium iodide at pH 7, and the fluorescence per cell was measured in a flow cytometer (*16*).

RESULTS

Susceptibility of CN and HC to External γ-Radiation

Both CN and HC were very resistant to the effects of γ-radiation, with 10% of the cells surviving at the doses of

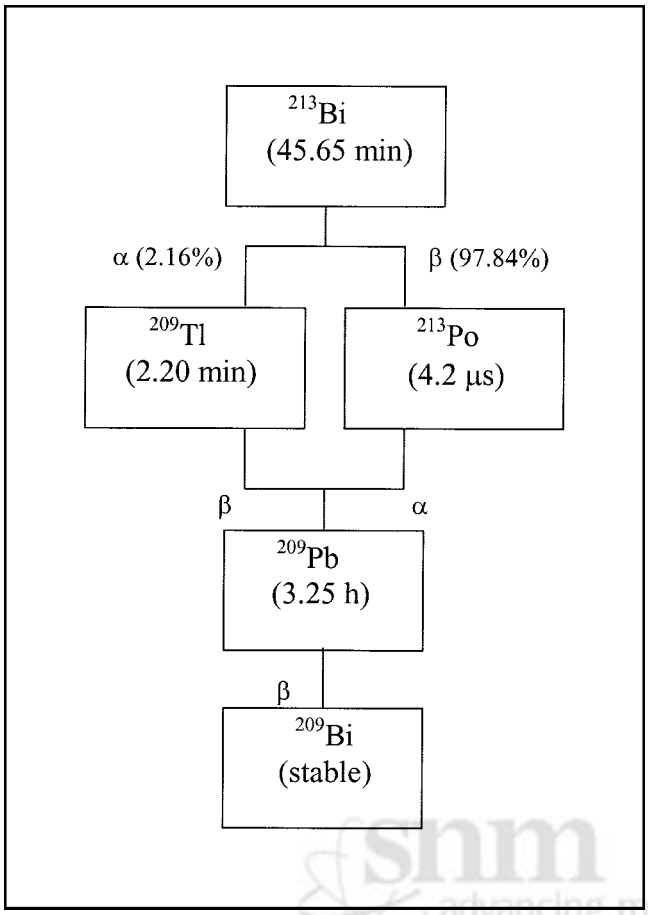

FIGURE 1. Decay series of ^{213}Bi. Physical half-lives and branching ratios are taken from (*13*).

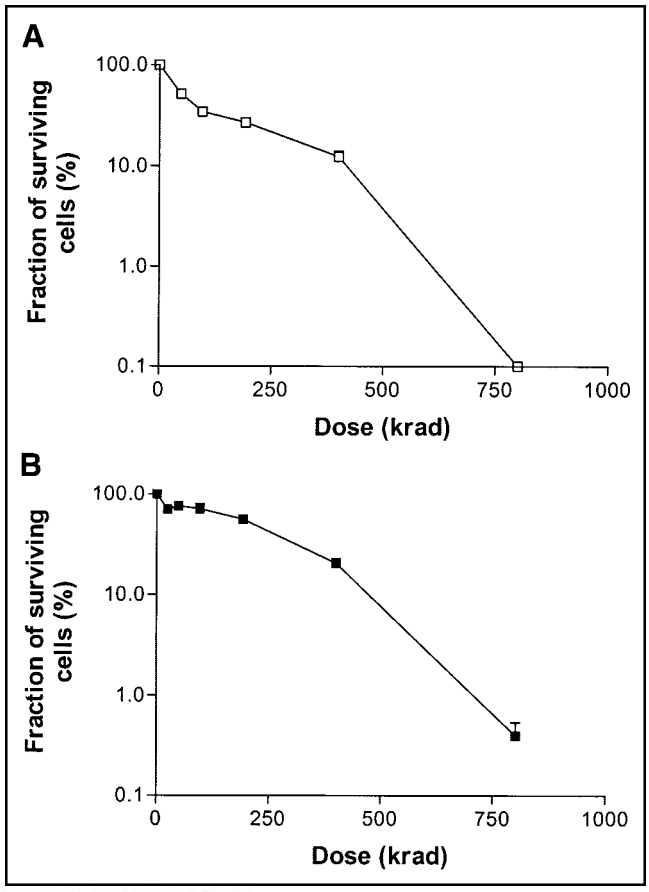

FIGURE 2. Cell survival of fungi after irradiation with external γ-rays (^{137}Cs source, 30 Gy/min [3,000 rad/min]). (A) CN. (B) HC.

~4,000 Gy (~ 400 krad), and <1% surviving a dose of ~8,000 Gy (~800 krad) (Figs. 2A and 2B).

Kinetics of Radiolabeled Antibodies Binding to CN and HC Cells

During the incubation period, the cellular uptake of radioactivity was linear up to 30 min for CN cells (Fig. 3). Some saturation in the cellular uptake was observed in the case of HC (Fig. 3). Least-squares fits of these data are provided in the caption for Figure 3. There was no measurable detachment of radiolabeled antibodies from the surface of the cells after incubation in PBS for either 1 or 48 h.

Cellular Dosimetry

CN, ^{188}Re-18B7 mAb. The incubation period $t_I = 30$ min, maintenance period $t_M = 48$ h = 2,880 min, and $t_{CF} = 2{,}880$ min. There was no cell division during t_I or t_M and, consequently, no biologic elimination occurred during these periods. Thus, substituting $k = 8.5 \times 10^{-8}$ min^{-1} and the various parameters into Equations 4 and 7 gives $\tilde{A}_I = (0.00226, s)A_o$ and $\tilde{A}_M = (0.189, s)A_o$. The CN cells form colonies through a budding process. Due to the nature of this budding process, which involves de novo synthesis of the polysaccharide capsule over the bud surface, one can assume that a negligible amount of radioactivity is passed from the parent cell to the daughter cells (*17*). Thus, it is reasonable to assume that all radioactivity remains with the parent cell. Furthermore, the capsule-bound antibody is located external to the cell wall and, consequently, is very unlikely to be internalized in significant quantities. Substituting the various parameters into Equation 9 gives $\tilde{A}_{CF} =$

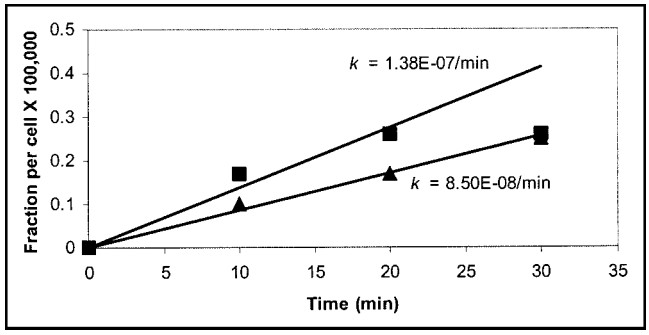

FIGURE 3. Kinetics of binding of ^{188}Re-18B7 mAb (▲) and ^{188}Re-9C7 mAb (■) to the cell surfaces of CN and HC, respectively. The ^{188}Re-18B7 mAb data are fitted to a linear function with a resulting slope of $k = 8.50\text{E}{-}08$ min^{-1}. The ^{188}Re-9C7 data up to a time of 20 min are similarly fitted with a resulting slope of $k = 1.38\text{E}{-}07$ min^{-1}. mAb 18B7 has IgG1 isotype, and 9C7 has IgM isotype.

(0.0267, s)A_o. Finally, as per Equation 2, the sum is $\tilde{A} = \tilde{A}_I + \tilde{A}_M + \tilde{A}_{CF} = 0.218\ A_o$ s. The absorbed dose is given by Equation 1, where $D_C = \tilde{A}\ S(C \leftarrow CS) = (0.218, s)A_o$ $(6.82 \times 10^{-05}, Gy\ Bq^{-1}\ s^{-1}) = 0.0149\ A_o$, Gy, where A_o is the kBq added to the tube of cells.

CN, ^{213}Bi-18B7 mAb. Substituting $k = 8.5 \times 10^{-8}\ min^{-1}$, $\lambda(^{213}Bi) = 1.52 \times 10^{-2}\ min^{-1}$, $t_I = 30$ min, $t_M = 60$ min, and $t_{CF} = 2{,}880$ min into Equations 4 and 7 gives $\tilde{A}_I = (0.00170, s)A_o$, $\tilde{A}_M = (0.00382, s)A_o$, and $\tilde{A}_{CF} = (0.00256, s)A_o$. The total cumulated activity $\tilde{A} = (0.00808, s)A_o$. Assuming that only the ^{213}Bi and ^{213}Po decays deposit energy in the cell and that ^{213}Po is in equilibrium with ^{213}Bi, Equation 1 gives $D_C = \tilde{A}\ (S(^{213}Bi,C \leftarrow CS) + 0.9784\ S(^{213}Po,C \leftarrow CS))$. Substitution of the S values gives $D_C = 0.0990$ Gy/kBq of ^{213}Bi added to the tube.

HC, ^{188}Re-9C7 mAb. Uptake of this mAb is reasonably linear up to 20 min; therefore, the dosimetry can be simplified by taking $t_I = 20$ min. The remaining 10 min of this period can then be added to the maintenance period so that $t_M = 2{,}890$ min. Finally, as before, $t_{CF} = 2{,}880$ min. There is no biologic elimination during t_I or t_M so only physical decay occurs during these periods. Substituting $k = 1.38 \times 10^{-7}\ min^{-1}$ (Fig. 2) and the various parameters into Equations 4 and 7 gives $\tilde{A}_I = (0.00164, s)A_o$ and $\tilde{A}_M = (0.207, s)A_o$. During cell division, which takes about 2 h, the membrane is shared between parent and daughter HC cells. Therefore, it is assumed that the bound radioactivity is divided between the 2 cells. Consequently, the effective half-time of the radioactivity in the cell during the colony-forming period is $\lambda_e = \lambda + \lambda_b = 6.8 \times 10^{-4}\ min^{-1} + 0.693/120\ min^{-1} = 0.00646\ min^{-1}$. Substituting the various parameters into Equation 9 gives $\tilde{A}_{CF} = (1.77 \times 10^{-4}, s)A_o$. Finally, as per Equation 2, the sum is $\tilde{A} = \tilde{A}_I + \tilde{A}_M + \tilde{A}_{CF} = 0.209\ A_o$ s. The absorbed dose is given by Equation 1, where $D_C = \tilde{A}\ S(C \leftarrow CS) = (0.209, s)\ A_o$ $(3.34 \times 10^{-4}\ Gy\ Bq^{-1}\ s^{-1}) = 0.0698\ A_o$, Gy, where A_o is the kBq added to the tube of cells.

Susceptibility of CN and HC to RIT with ^{188}Re- and ^{213}Bi-Radiolabeled Antibodies

The RIT of CN cells was performed with both ^{188}Re- and ^{213}Bi-radiolabeled antibodies. The dependence of the RIT-treated CN and HC cell survival on the amount of radioactivity added to the cells and the cellular absorbed dose delivered by radiolabeled antibodies is presented in Figure 4. Control experiments demonstrated that unlabeled mAbs in either prereduced form or conjugated with CHXA″ did not cause the death of fungal cells. Interestingly, in the case of CN, the doses of ~2 and 1 Gy (~0.2 and 0.100 krad) for ^{188}Re-18B7 and ^{213}Bi-18B7, respectively, eradicated >99% of CN cells (Figs. 4A and 4B). In contrast, radiolabeled control antibody MOPC21 with the same specific activity produced only minimal killing within the investigated range of doses ($P < 0.001$). The significantly higher killing associated with the specific antibody almost certainly reflects higher radiation exposure for CN as a consequence of antibody binding to the CN capsule.

Flow Cytometry

It is known that exposure to particulate radiation can cause cell cycle arrest and apoptotic death in mammalian cells (*18,19*), thus contributing to the efficacy of RIT. We have performed flow cytometry analysis of irradiated fungal cells to establish whether exposure of fungal cells to radiation caused an apoptosis-like death in unicellular organisms. The results of flow cytometry analysis of apoptosis in nonirradiated cells, cells irradiated with γ-rays, and cells treated with RIT are presented in Table 1 for both CN and HC. Exposure of fungal cells to 4,000 Gy (400 krad) of γ-radiation caused an apoptosis-like phenomenon in a significant percentage of CN and HC cells. Strikingly, RIT of CN with ^{213}Bi-18B7 caused 80%–90% of the cells to die through an apoptotic-like pathway, which is consistent with the very low survival rate of ^{213}Bi-18B7–treated cells at the highest dose of 11.7 Gy (1.17 krad) (Fig. 4B).

DISCUSSION

The field of classical radiobiology is almost 100 y old (*20*), and the basic concepts of the effects of external radiation on cells have been studied extensively. In contrast, the field of RIT has emerged during the last 2 decades and is a field in flux with the introduction of new techniques resulting in a rapidly evolving discipline (*21*). In fact, it has been pointed out that the classical concepts of radiobiology often cannot explain the effectiveness of RIT in laboratory and clinical experimentation (*20*). Recently we observed remarkable microbicidal effects when a radiolabeled antibody was incubated with CN in vitro and when the radiolabeled antibody was used to treat cryptococcal infection in vivo (*7*). That result led us to revisit the susceptibility of fungal cells to radiation and to formally compare the results obtained for external γ-radiation and RIT using well-established methods of dosimetry.

Comparing the response of CN and HC fungi to γ-radiation and RIT with ^{188}Re- and ^{213}Bi-labeled antibodies revealed that RIT with organism-specific radiolabeled antibodies (Figs. 4A and 4B) was significantly more efficient in mediating fungal cell killing than exposure to external γ-radiation (Fig. 1). The radiation doses required to kill CN fungal cells were 1,000-fold lower when delivered by radiolabeled antibodies compared with γ-radiation and, consistent with classical radiobiology, the α-emitter ^{213}Bi was about 2 times more lethal than the β-emitter ^{188}Re, presumably due to the emission of high linear-energy-transfer α-particles that can kill a cell with 1 or 2 hits. The dose from α-particles constitutes >97% of the cellular absorbed dose from ^{213}Bi.

Furthermore, the dose rates delivered by the radiolabeled antibodies were thousands of times lower than those delivered acutely with the external γ-irradiation (30 Gy/min). This is consistent with clinical RIT, where peak dose rates

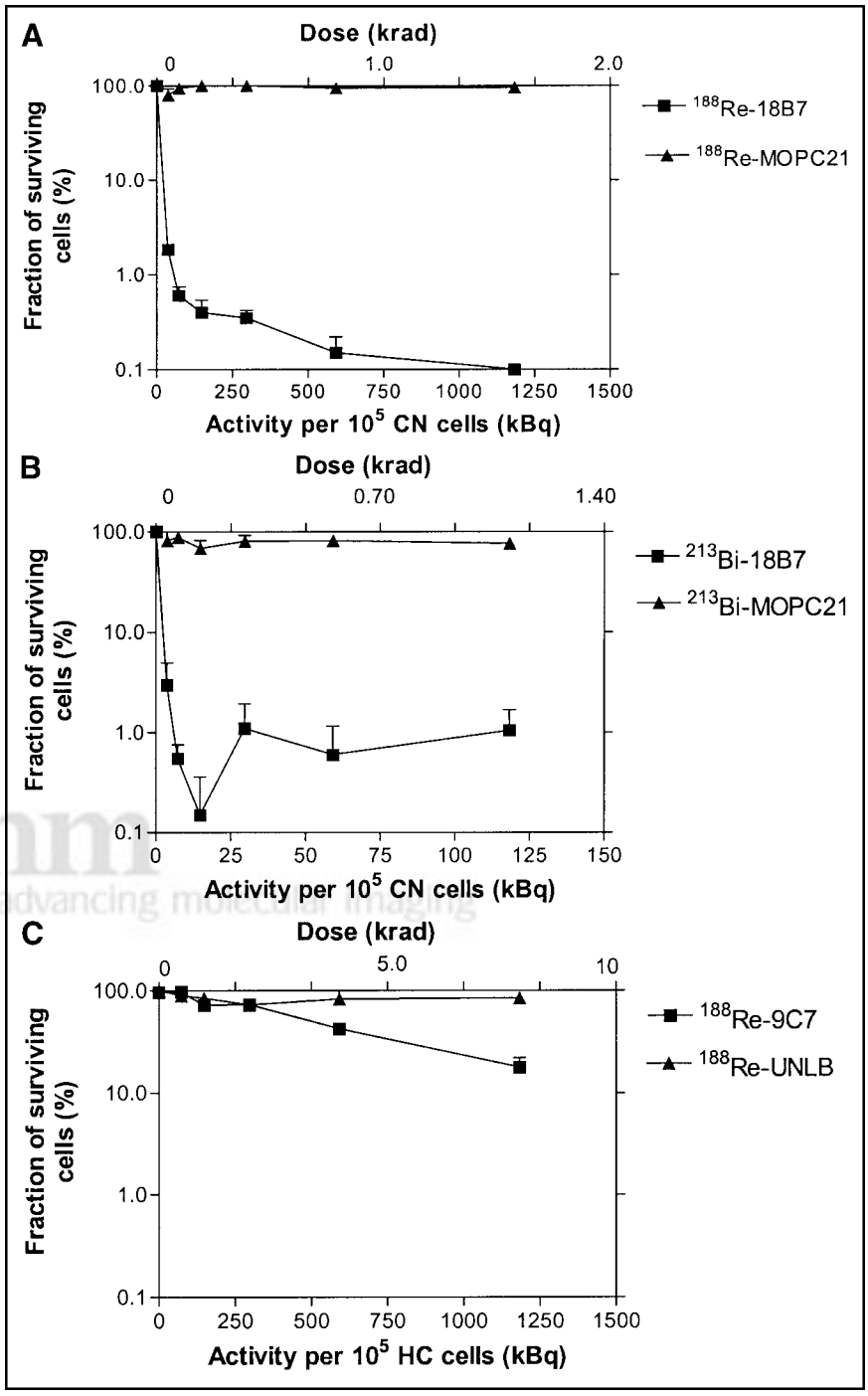

FIGURE 4. Cell survival of fungi after RIT of CN with ^{188}Re-18B7 mAb (A), CN with ^{213}Bi-18B7 mAb (B), or HC with ^{188}Re-9C7 mAb (C).

of 0.1 Gy/h (10 rad/h) (*18*) are observed. For comparison, high-dose rate radiation, which is typical for external beam radiation therapy, delivers 60 Gy/h (6 krad/h). Thus, from the viewpoint of radiation therapy, RIT delivers suboptimal dose rates to tumors (or to microbial cells in our case). However, despite this, targeted radionuclide therapy can be effective through mechanisms such as the induction of apoptosis in irradiated cells, "bystander" effect (death of adjacent, nonirradiated cells), and cell cycle arrest (*18*,*19*,*22*,*23*). The flow cytometry results in Table 1 indicate that low doses of particulate radiation delivered by organism-specific antibody caused the majority of treated cells to die by an apoptotic-like mechanism, whereas much higher doses of γ-radiation induced this apoptosis-like phenomenon only in ~30% of irradiated cells. These results are consistent with and supportive of the fact that RIT using α- and β-emitters is effective despite radiation doses that seem inadequate for microbicidal effects according to standard cellular dosimetry calculations. The surprising efficacy of RIT could reflect the occurrence of a different cascade of

TABLE 1
Induction of Apoptosis-Like Phenomenon in CN and HC Cells by RIT and γ-Radiation

Fungus	Radiation type	Cellular absorbed dose	Apoptotic-like cells (%)
CN	—	0	3.8
CN	γ-Rays	1,000 Gy (100 krad)	13
CN	γ-Rays	2,000 Gy (200 krad)	24
CN	γ-Rays	4,000 Gy (400 krad)	37
CN	—	0	19
CN	β-Particles ^{188}Re-18B7, 1,184 kBq (32 μCi)	18 Gy (1.8 krad)	43
CN	α-Particles ^{213}Bi-18B7, 118 kBq (3.2 μCi)	12 Gy (1.2 krad)	81
HC	—	0	12
HC	γ-Rays	4,000 Gy (400 krad)	18
HC	—	0	14
HC	β-Particles ^{188}Re-9C7, 1,184 kBq (32 μCi)	84 Gy (8.4 krad)	41

CN cultures were grown for 5 d before treatment; HC cultures were grown for 7 d before treatment.

events resulting from the interaction of the electrons and α-particles (emitted by radiolabeled antibodies on the cell surface) with the cell membrane itself or with structures within the cell. In fact, there is new evidence to suggest that cellular radioactivity can cause uniquely different gene expression profiles than external γ-rays (*24*). Alternatively, it is conceivable that antibody molecules also mediate local effects that enhance the microbicidal efficacy of radiation. In this regard, antibody molecules have recently been shown to be catalysts for the production of microbicidal oxygen-related oxidants (*25*). Hence, particulate radiation combined with local production of toxic oxidative molecules could result in synergistic antimicrobial effects that translate into significantly greater efficacy for RIT than would be expected from dosimetry calculations alone.

Despite the fact that HC and CN responded similarly to external γ-radiation (Figs. 2A and 2B), HC was less susceptible to the RIT with ^{188}Re-labeled specific antibody than CN (Figs. 4A and 4C). A cellular absorbed dose of about 80 Gy (8 krad) was required to eradicate 80% of HC cells (Fig. 4C), whereas <1 Gy was required to eradicate a similar fraction of CN cells (Fig. 4A). The difference in calculated cellular absorbed doses is, in part, attributed to their very different cell diameters (5 μm for HC and 10 μm for CN), which in turn affects their mean absorbed dose per decay (S value). Although the cellular absorbed doses required to eradicate a specific fraction of cells may be quite different, it is possible that the local absorbed doses on the cellular membrane are more similar. Another factor that may be involved in the observed differences in responses is a lower density of antibody molecules on the surface of HC cells relative to CN cells. For CN, $\sim 10^8$ antibody molecules were bound to each cell whereas, for HC, only 2×10^7 antibody molecules were bound to each cell. Furthermore, the fact that the mAbs used in CN and HC RIT are of IgG1 and IgM isotypes, respectively, raises the possibility that isotype-related effects contribute to the lethality of RIT. Nevertheless, regardless of the mechanisms involved, the cellular absorbed dose of 80 Gy (8 krad) delivered by ^{188}Re-9C7 mAb was almost 100 times lower than the dose of γ-radiation needed to cause comparable killing of HC cells (Figs. 1B and 4C). The killing was antibody-specific since incubation of HC with radiolabeled control antibody resulted in only negligible killing of HC cells.

CONCLUSION

Our results demonstrate that particulate radiation delivered by organism-specific radiolabeled antibodies is significantly more efficient in killing the human pathogenic fungi CN and HC than external γ-radiation. The results provide strong experimental support for the concept of using RIT as an antimicrobial modality. The mechanism for this effect is uncertain but may involve a cascade of different events that ultimately lead to cell death by apoptosis. These results suggest that the relative sensitivity of fungi toward particulate radiation delivered by specific radiolabeled antibodies can be used for designing novel therapeutic strategies against difficult-to-treat fungal infections.

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