MAJOR ARTICLE

Interaction of Radiolabeled Antibodies with Fungal Cells and Components of the Immune System In Vitro and during Radioimmunotherapy for Experimental Fungal Infection

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Background. The usefulness of radioimmunotherapy (RIT) for infectious diseases was recently demonstrated for several fungal and bacterial infections, but the mechanisms by which RIT is effective against microbes are uncertain.

Methods. We investigated the interaction between polysaccharide capsule–binding 18B7 monoclonal antibodies (MAbs) labeled with α-emitter ^{213}Bi and *Cryptococcus neoformans* cells as well as between ^{213}Bi-18B7 and components of immune system, both in vitro and in vivo.

Results. For ^{213}Bi-18B7, the microbicidal effect was predominantly due to "direct-hit" killing, with some contribution from the "crossfire" effect. The efficacy of cell killing correlated with the binding capacity of the MAb to the capsule and was dependent on the MAb isotype. RIT also promoted the apoptosis-like death of fungal cells. Cooperation was observed in vitro between the antifungal activity of macrophages and RIT, suggesting the potential for synergistic action in vivo. RIT was associated with changes in concentration of the cytokines interleukin (IL)–2, IL-4, IL-10, tumor necrosis factor–α, and interferon-γ, suggesting that the therapeutic effects of RIT may result from changes in the inflammatory response.

Conclusions. The present results suggest that the antimicrobial efficacy of RIT involves killing through promotion of fungal cell apoptosis-like death, reduction in yeast capsule size, cooperation with macrophages, and modulation of the inflammatory response.

There is an urgent need for new approaches to the treatment of infectious diseases [1]. One novel approach that has the potential for general applicability across pathogen classes is radioimmunotherapy (RIT) [2]. RIT takes advantage of the specificity of antigen-antibody interaction to deliver cytotoxic radiation to the target, mediating an antitumor or antimicrobial effect. Two RIT-based cancer therapies are presently licensed, with more in development [3]. Radiolabeled monoclonal antibodies (MAbs) have prolonged survival and reduced fungal burden in the organs of mice infected systemically with *Cryptococcus neoformans* [2] and *Streptococcus pneumoniae* [4].

The mechanism by which RIT is effective in murine cryptococcosis [2] is uncertain. In oncology, the mechanisms of RIT are still being debated [5]. The major radiobiological mechanisms of cancer RIT are considered to be the "direct-hit" effect and the "crossfire" effect (also referred to as the "bystander" effect), both of which can promote apoptosis and cell cycle redistribution [6, 7]. Labeling of a nonmicrobicidal *C. neoformans*–specific MAb with ^{213}Bi, a radioisotope that emits α-particles (which are He atoms with the charge of +2 and mass of

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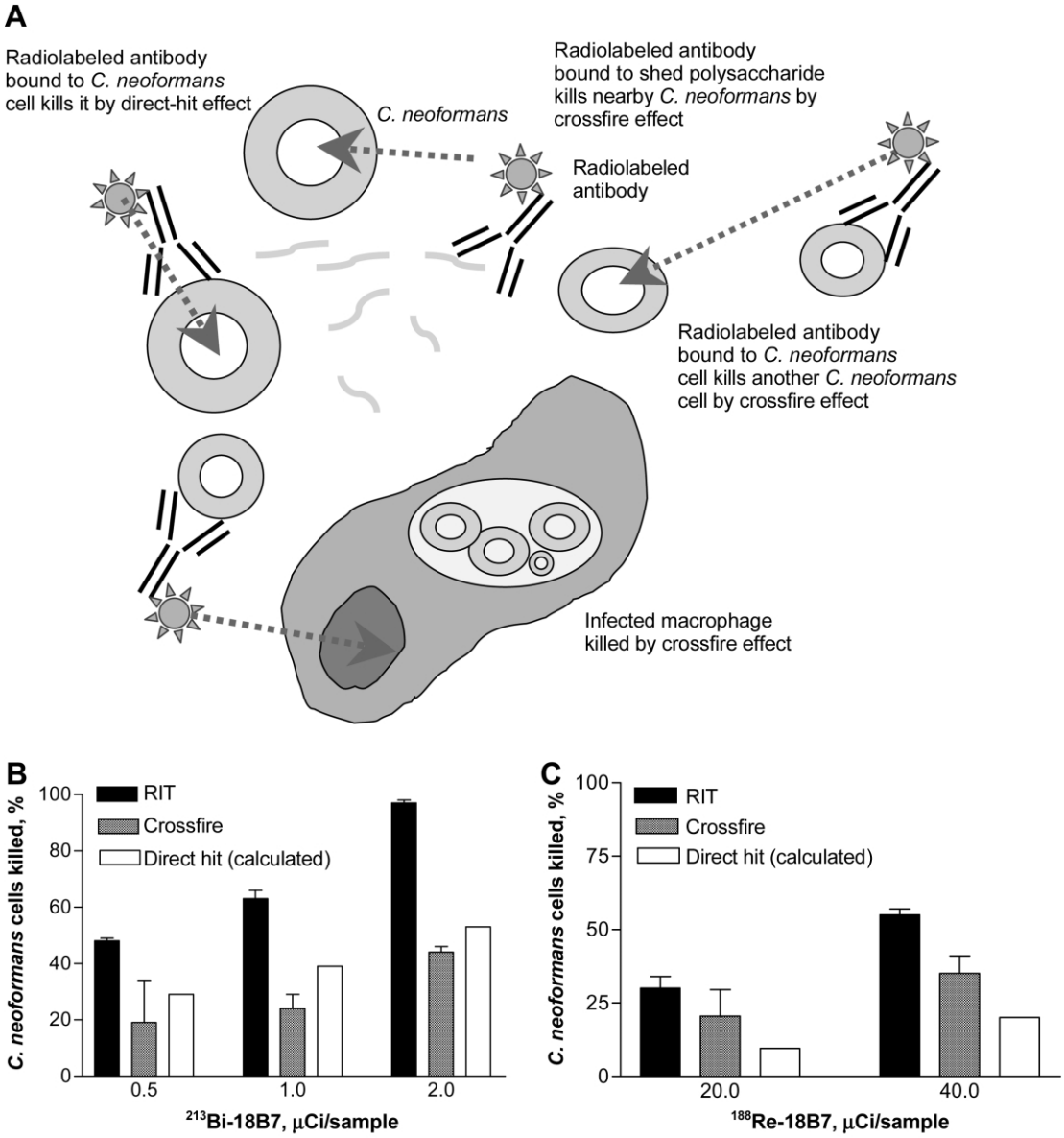

Figure 1. Contribution of different radiobiological effects to radioimmunotherapy (RIT) with ^{213}Bi-18B7 and ^{188}Re-18B7 monoclonal antibodies (MAbs) for *Cryptococcus neoformans* infection. *A,* Schematic of killing of *C. neoformans* and *C. neoformans*–infected cells by a radiolabeled MAb. *B* and *C,* Percentage of killing of *C. neoformans* by ^{213}Bi-18B7 and ^{188}Re-18B7 MAbs, respectively. The contribution of the direct-hit effect toward cell killing was calculated by subtracting the percentage of cells killed by the crossfire effect from the percentage of cells killed by RIT. All experiments were performed at least 2 times, with all samples prepared in duplicate; data shown are means ± SDs. For both ^{213}Bi and ^{188}Re, there was a significant difference ($P < .02$) between the percentage of cells killed by RIT and by the crossfire effect.

4) or ^{188}Re, a radioisotope that emits high-energy electrons, converted the MAb into a microbicidal molecule [2], suggesting that the efficacy of RIT involved fungicidal effects. However, radiation may also kill the macrophages in which *C. neoformans* reproduces. In this regard, depletion of neutrophils improved survival in murine pulmonary cryptococcosis [8].

We hypothesized that fungal cells could be killed by antibody-delivered radiation via the direct-hit effect (killing of a cell by radiation emanating from a radiolabeled molecule bound to this cell), the crossfire effect (killing of a cell by radiation emanating from a radiolabeled molecule bound to an adjacent or distant cell) [3, 6, 9], or a combination of both (figure 1*A*). These terms are defined by the nuclear medicine field. We considered several potential mechanisms for RIT's action, including production of reactive oxygen species (ROS) [10], synergy with macrophages, and effects on the inflammatory re-

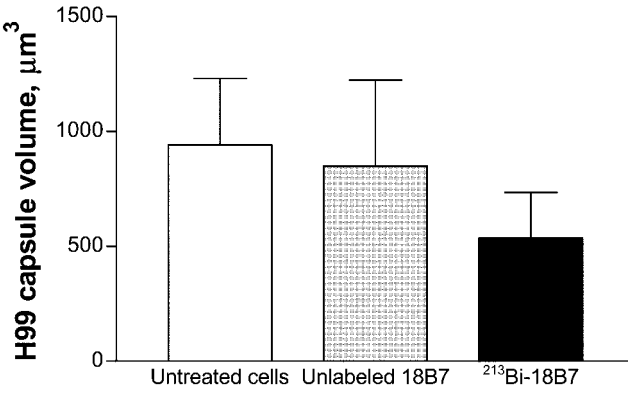

Figure 2. Change in H99 capsule volume after treatment with ^{213}Bi-18B7 monoclonal antibody (MAb) or matching amounts of unlabeled 18B7. H99 cells were grown overnight in Sabouraud dextrose broth at 30°C and subcultured into capsule-inducing medium (10% Sabouraud dextrose broth in 50 mmol/L HEPES). Control cells were left untreated, and 3×10^6 cells/sample were treated with 2 μCi/μg ^{213}Bi-18B7 or with the matching amount of unlabeled 18B7. Control and treated cells were stained with India ink and observed under an Olympus AX70 microscope. The equation $(4\pi/3)(D/2)^3$, where D is the diameter of the capsule and cell body, was used for calculation of the cell volumes. All experiments were performed at least 2 times, with all samples prepared in duplicate; data shown are means ± SDs. The reduction in capsule size was significant with ^{213}Bi-18B7 ($P < .001$) and was not significant with unlabeled 18B7 ($P = .2$).

sponse [8, 11]. Here, we report that RIT has several potential mechanisms of action against *C. neoformans*.

METHODS

C. neoformans. *C. neoformans* strains H99 *(C. neoformans* var. *grubii)* and 24067 *(C. neoformans* var. *neoformans)* were obtained from J. Perfect (Duke University) and the American Type Culture Collection, respectively. The cells were grown as described elsewhere [12].

C. neoformans–*specific MAbs and radiolabeling of MAbs.* Glucuronoxylomannan-binding murine IgG1 MAb 18B7 was produced as described elsewhere [13]. The isotype-matching control MAb MOPC21 was acquired from MP Biochemicals. A family of 3E5 MAbs with identical V regions but different isotypes (IgG1, IgG2a, IgG2b, and IgG3) has been described elsewhere [14]. Actinium (^{225}Ac) for construction of ^{225}Ac/^{213}Bi generators was produced at the Institute for Transuranium Elements (Karlsruhe, Germany). Radiolabeling of MAbs with ^{213}Bi and ^{188}Re eluted from ^{188}Re/^{188}W generator (Oak Ridge National Laboratory, Oak Ridge, TN) was performed as described elsewhere [2].

In vitro killing of* C. neoformans *cells by ^{213}Bi-18B7 and ^{188}Re-18B7 MAbs. For RIT with ^{213}Bi, 5×10^6 live cells were incubated with either labeled or unlabeled MAbs (0.2–4.0 μg/mL) for 0.5 h at 37°C, collected by centrifugation, incubated in PBS at 37°C for 3 h, treated with Tween 80 (0.5%), triturated 20 times, diluted, and plated for counting of colonies. For crossfire experiments, 5×10^6 heat-killed (65°C for 1 h) *C. neoformans* cells were incubated with either labeled or unlabeled MAb (0.2–4.0 μg/mL) for 0.5 h at 37°C, collected by centrifugation, and mixed with 5×10^6 live cells; the mixture was incubated for 3 h at 37°C, treated with Tween 80, diluted, and plated. The direct-hit and crossfire RIT experiments with ^{188}Re were conducted in essentially the same manner, except that *C. neoformans* cells were incubated for 48 h at 4°C, to prevent cell division before dilution and plating.

Interaction of ^{213}Bi-18B7 MAb with the* C. neoformans *capsule. Treatment of H99 cells grown in capsule-inducing medium (hereafter, "induced H99 cells") with ^{213}Bi-18B7 MAb was performed as described above, and the cell volume was measured as described elsewhere [12].

Binding of 18B7 MAb to H99 and 24067 cells. ^{188}Re-18B7 was added in different amounts to 24067, H99, or induced H99 cells (2×10^6 cells/tube). After incubation for 1 h at 37°C, the samples were counted in a gamma counter, the cells were collected by centrifugation, and the pellets were counted. Scatchard analysis was used to compute the MAb affinity constant and the number of binding sites per cell, as described elsewhere [15].

Dependence of MAb binding to* C. neoformans *capsule and* C. neoformans *killing on MAb isotype. Binding of ^{213}Bi-3E5 MAbs to *C. neoformans* 24067 cells was performed as described above. The aliquots of cells treated with each of the labeled 3E5 MAbs were also tested for efficiency of *C. neoformans* killing.

Radiation-triggered generation of ROS. Indigo carmine bleaching was done as described elsewhere [10], with minor modifications. Approximately 2.5×10^6 *C. neoformans* cells were suspended in 10 mmol/L indigo carmine, and ^{213}Bi-18B7 (0.5 or 2.3 μCi/1×10^5 cells), irrelevant MAb ^{213}Bi-MOPC21 (2.3 μCi/1×10^5 cells), or unlabeled 18B7 (4.0 μg/mL) was then added. The absorbance of the reaction mixture at 610 nm was measured at 20-min intervals for 90 min. To provide a control, the cells were mixed with unlabeled 18B7 MAb, and the mixture was subjected to 254-nm UV light.

Alternatively, we used an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes), which detects H_2O_2. MAb ^{213}Bi-18B7 (0.5–8.1 μCi/1×10^5 cells) was added to 5×

Table 1. Calculated nos. of binding sites per cell and affinity constants for 18B7 monoclonal antibody binding to various *Cryptococcus neoformans* strains.

Strain	Binding sites/cell, no.	Affinity constant, $(mol/L)^{-1}$
24067	1.1×10^6	1.7×10^9
H99	1.2×10^6	5.9×10^8
Induced H99[a]	1.1×10^6	2.7×10^8

[a] H99 cells grown in capsule-inducing medium.

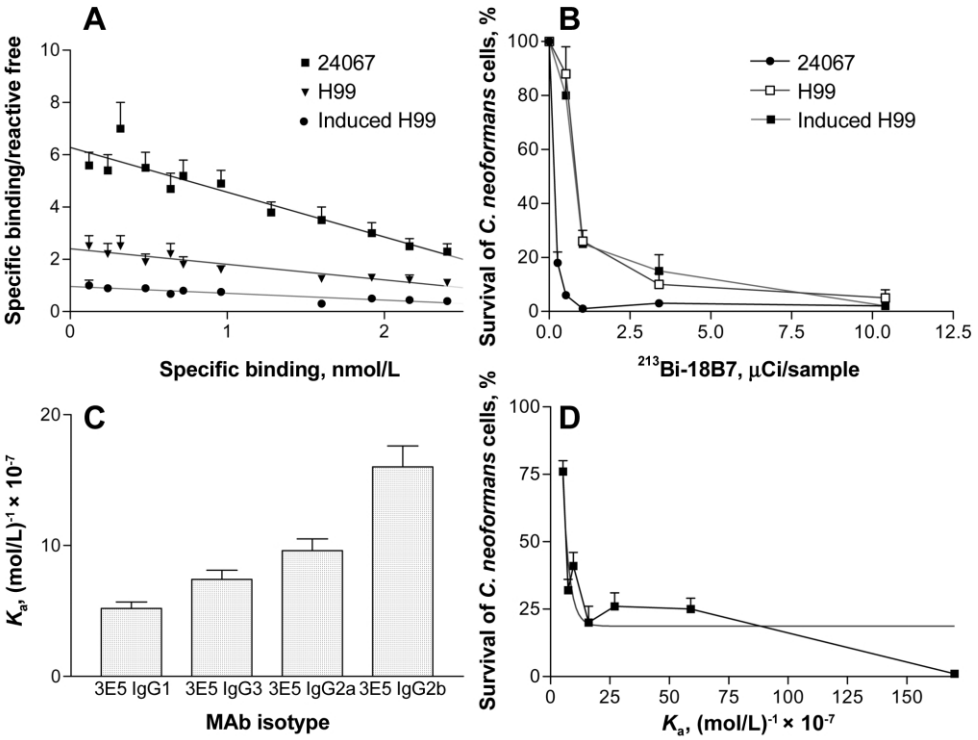

Figure 3. Dependence of monoclonal antibody (MAb) binding to *Cryptococcus neoformans* cells and their killing by radiolabeled MAbs on strain type and MAb isotype. *A,* Scatchard plot of ^{188}Re-18B7 binding to 24067, H99, and induced H99 cells. *B,* Radioimmunotherapy (RIT) with MAb ^{213}Bi-18B7 for 24067, H99, and induced H99 cells. *C,* Affinity constants (K_a) for the 3E5 MAb family. *D,* Dependence of *C. neoformans* cell killing on the MAb affinity constant. Data points 1–4 (left to right) are for ^{213}Bi-3E5 MAbs and 24067 cells; data points 5 and 6 are for ^{213}Bi-18B7 and H99 (point 5) and induced H99 (point 6) cells; and data point 7 is for ^{213}Bi-18B7 and 24067 cells. All cells (1×10^5/sample) were treated with 1.0 μCi of ^{213}Bi-labeled MAb. Data were fitted into the 1-phase exponential decay equation $Y = 398e^{-0.38x} + 18.71$ ($R^2 = 0.8$) using nonlinear regression *(solid line).* All experiments were performed at least 2 times, with all samples prepared in duplicate; data shown are means ± SDs. There was a significant difference ($P < .02$) between affinity constants for the 3E5 MAb family.

10^6 *C. neoformans* cells, followed immediately by the Amplex Red reagent and horseradish peroxidase in the reaction buffer. The absorbance of the reaction mixture at 540 nm was measured at 30, 50, and 85 min, and the amount of H_2O_2 produced was calculated.

Quantification of the apoptosis-like death of* C. neoformans *cells. Either 5 mmol/L H_2O_2 (apoptosis-positive control) or 5 mmol/L ^{213}Bi-18B7 (0.6–2.4 μCi/1×10^5 cells) was added to 2×10^6 *C. neoformans* cells, and the mixture was incubated for 3 h at 37°C, followed by an additional 3 h at room temperature. Untreated *C. neoformans* cells served as an apoptosis-negative control. The cells were subsequently stained with sulforhodaminyl-L-valylalanylaspartyl fluoromethyl ketone, which binds to all caspases, and fixed using a FLICA (fluorochrome-labeled inhibitors of caspases) kit (Immunochemistry Technologies), in accordance with the manufacturer's instructions. Fluorescence was measured using a Becton Dickinson FACScan.

Alternatively, the APO-BrdU TUNEL assay kit (Molecular Probes) was used in accordance with the manufacturer's instructions. This method detects DNA fragments. Apoptosis-positive *C. neoformans* cells and apoptosis-negative control cells were prepared as described above. Also, the negative and positive controls (fixed human lymphoma cells) included in the kit were used for assay validation. The samples were stained with AlexaFluor 488 dye–labeled anti-BrdU antibody and propodium iodide and analyzed by flow cytometry.

Macrophage experiments. Murine macrophage–like J774.16 cells were cultured and maintained as described elsewhere [15]. The viability of macrophages treated with 0–10 μCi of ^{213}Bi-18B7 for 3 h at 37°C was measured by trypan blue dye exclusion assay. To evaluate the interaction of RIT with macrophages, 1×10^5 macrophages/well were plated on a 96-well plate and stimulated overnight with 500 U of interferon (IFN)–γ [16]. The medium was replaced with a suspension of 2×10^4 *C. neoformans* cells in fresh medium, giving a 10:1 effector-to-target cell ratio. *C. neoformans* cells were treated with different amounts of unlabeled 18B7 or with matching amounts of ^{213}Bi-18B7. After incubation for 3 h at 37°C, the supernatants from each well were collected, lysed, and plated for determination of colony counts. The effect of the medium on RIT was studied by performing

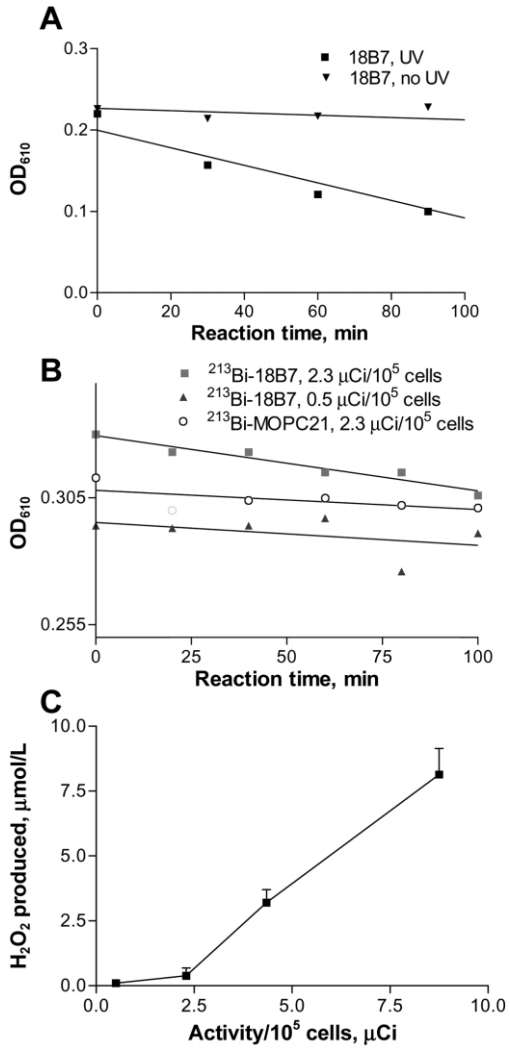

Figure 4. Detection of reactive oxygen species (ROS) in the presence of *Cryptococcus neoformans* 24067 cells and 18B7 monoclonal antibody (MAb) by indigo carmine bleaching and Amplex Red assay. *A,* Indigo carmine detection of UV-triggered formation of ROS. *B,* Indigo carmine detection of radiation-triggered formation of ROS. ^{213}Bi-MOPC21 is an irrelevant MAb. *C,* Detection of H_2O_2 in ^{213}Bi-18B7–treated *C. neoformans* samples by use of an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. All experiments were performed at least 2 times, with all samples prepared in duplicate; data shown are means ± SDs.

RIT in PBS, Dulbecco's modified Eagle medium (DMEM), and mouse serum.

Cytokine analysis. All animal experiments were done in accordance with the guidelines of the Albert Einstein College of Medicine Institute for Animal Studies. Three groups of 10 AJ/Cr mice were infected intravenously with 1×10^5 *C. neoformans* H99 or 24067 cells. Twenty-four hours after infection, the mice either were left untreated or were treated intraperitoneally with 150 μCi of ^{213}Bi-18B7 (30 μg/mouse) or 30 μg of unlabeled 18B7. For the H99-infected mice, 5 from each group were killed at 48 h, and the fungal burden in their lungs and brains was analyzed. The remaining 5 mice in each group were killed on day 7 after infection, and their lungs and brains were homogenized in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim) for cytokine analysis. The supernatants were tested for interleukin (IL)–2, IL-4, IL-10, tumor necrosis factor (TNF)–α, and IFN-γ by use of ELISA kits (BD PharMingen).

Flow-cytometric analysis of lung leukocytes. Three groups of 5 AJ/Cr mice were infected intravenously with *C. neoformans* 24067 and either left untreated or treated (as described above) with ^{213}Bi-18B7 or unlabeled 18B7. On day 7 after infection, lungs were removed, homogenized, and digested enzymatically, and the cell suspension was collected by centrifugation as described elsewhere [8, 11]. For flow-cytometric analysis, the following MAbs were used: R-phycoerythrin–labeled anti-CD45, fluorescein isothiocyanate–labeled anti–mouse CD4, and Cy-Chrome-labeled anti–mouse CD8 (BD PharMingen). The samples were analyzed using a Becton Dickinson FACScan. Controls consisted of isotype-matched irrelevant MAbs.

Statistical analysis. Student's *t* test for unpaired data was used to analyze differences in cytokine expression and the number of inflammatory cells between the treatment groups and to compare the results of the repeat experiments. Differences were considered to be statistically significant if $P < .05$. The Scatchard binding and ROS data were analyzed by linear and nonlinear regression (Prism; version 2; GraphPad).

RESULTS

Killing of* C. neoformans *by ^{213}Bi-labeled MAb through both direct-hit and crossfire effects. To elucidate the contribution of direct-hit and crossfire effects to RIT for *C. neoformans* infection, we compared the fungicidal activity of MAb conjugated to ^{213}Bi and ^{188}Re, isotopes with different emission ranges in tissue (50–80 μm for ^{213}Bi vs. 10 mm for ^{188}Re). In cancer RIT, it is assumed that ^{213}Bi kills through direct-hit effects and that ^{188}Re kills through crossfire effects [3, 17, 18]. In principle, every cell with bound radiolabeled MAb molecules can be killed by a direct hit and simultaneously serve as a source of crossfire radiation. By measuring the killing of cells during RIT and in crossfire experiments, we can calculate the contribution of direct-hit effects toward cell killing by subtracting the percentage of cells killed by crossfire effects from the percentage of cells killed by RIT.

To observe crossfire, we had to ensure that the cells that served as the sources of crossfire radiation could not themselves be killed by direct hit. Consequently, we used heat-killed *C. neoformans* cells. Experiments with ^{213}Bi-18B7 showed that, although most kills were due to direct hits, the crossfire effect also contributed to the fungicidal effect of RIT (figure 1*B*). No killing of *C. neoformans* cells by unlabeled 18B7 was observed. We have previously shown that radiolabeled irrelevant MAbs

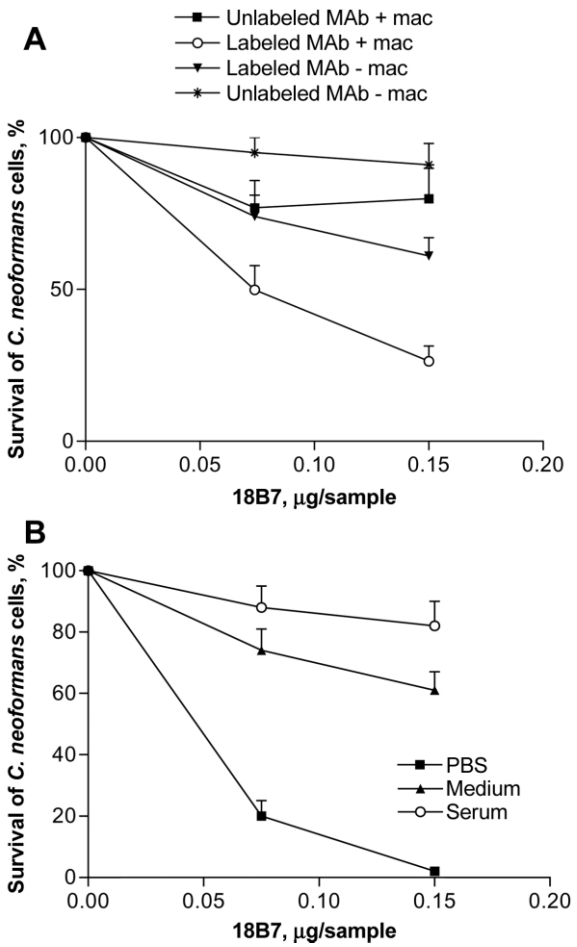

Figure 5. Cooperation of macrophages in the killing of *Cryptococcus neoformans* cells. *A,* Killing of 24067 cells by macrophage-like J774.16 cells (mac) in the presence of ^{213}Bi-18B7 monoclonal antibody (MAb). Labeled MAb without J774.16 cells, unlabeled MAb without J774.16 cells, and unlabeled MAb with J774.16 cells were used as controls. All experiments were performed in J774.16 cell medium. *B,* Killing of 24067 cells by ^{213}Bi-18B7 MAb alone in different media. The effect of the medium on radioimmunotherapy was studied by performing it in PBS, Dulbecco's modified Eagle medium, and mouse serum. All experiments were performed at least 2 times, with all samples prepared in duplicate.

were unable to kill *C. neoformans* cells [2]. For ^{188}Re-18B7, the crossfire effect was responsible for most of the *C. neoformans* killing (figure 1*C*).

Reduction of capsule size by treatment of* C. neoformans *capsule with capsule-binding ^{213}Bi-18B7 MAb. Incubation of *C. neoformans* with ^{213}Bi-18B7 resulted in a significant reduction ($P < .001$) in capsule size (figure 2). This effect was not observed with unlabeled 18B7.

Effect of strain and MAb isotype on capsule binding and cell killing by RIT. We measured pronounced differences in the abilities of 24067, H99, and induced H99 cells to bind to 18B7 MAb (figure 3*A*) and found lower affinity constants for 18B7 MAb binding to H99 and induced H99 cells than for binding to 24067 cells (table 1). Strains 24067 and H99 differed significantly in their susceptibility to RIT (figure 3*B*). The ^{213}Bi-3E5 MAbs bound differently to 24067 cells (figure 3*C*), with 3E5 IgG1 and 3E5 IgG2b having the lowest and highest affinity constants, respectively. The killing of *C. neoformans* cells by ^{213}Bi-labeled MAbs was proportional to their affinity constants (figure 3*D*) and was described by the 1-phase exponential decay equation $Y = 398e^{-0.38x} + 18.71$ ($R^2 = 0.8$).

Induction of the formation of ROS by radiation. Exposing the mixture of *C. neoformans* cells and unlabeled 18B7 to UV light initiated the formation of singlet molecular oxygen ($^1O_2{}^*$), which is required for MAb-catalyzed production of ROS [10] (figure 4*A*). Radiation from radiolabeled MAb in solution, which causes the hydrolysis of water, also triggered the antibody-catalyzed generation of ROS. For ^{213}Bi-18B7, ROS formation was dose dependent, with ROS formation observed at 2.3 μCi/1 $\times$ 10^5 cells and no ROS formation for 0.5 μCi/1 $\times$ 10^5 cells (the slope was not significantly different from zero; $P = .37$) (figure 4*B*). No ROS formation was observed for the control ^{213}Bi-MOPC21 ($P = .20$), suggesting a need for antigen-antibody interaction in this process (figure 4*B*). The indigo carmine bleaching technique was validated by use of Amplex Red reagent, which in the presence of peroxidase reacts with H_2O_2 to produce red-fluorescent resorufin [19]. The amount of H_2O_2 produced was clearly dependent on the level of radioactivity in the sample (figure 4*C*).

Induction of apoptosis-like death in* C. neoformans *cells by RIT. Environmental stresses, antibiotics, and UV light can induce apoptosis-like death in fungal cells [20–22]. RIT caused a significant percentage of *C. neoformans* cells to undergo apoptosis-like death, as determined by FLICA assay [23], and the effect was dose dependent, with a mean $\pm$ SD of 8% $\pm$ 3% and 18% $\pm$ 5% of cells demonstrating apoptosis-like changes after treatment with 0.6 μCi and 1.2 μCi of ^{213}Bi-18B7, respectively ($P = .03$). Treatment of *C. neoformans* cells with 5 mmol/L H_2O_2 resulted in 20% of cells demonstrating apoptosis-like changes, which matches published results for *Candida albicans* [20]. At 2.4 μCi/1 $\times$ 10^5 cells, the percentage of cells demonstrating apoptosis-like changes remained the same (mean $\pm$ SD, 18% $\pm$ 6%; $P = .9$), possibly because some treated cells became necrotic. When cells are exposed to high concentrations of apoptosis-causing agents (such acetic acid and H_2O_2), necrotic cells can outnumber apoptotic cells, which results in reduced staining [20]. FLICA data was validated by the TUNEL technique, using the APO-BrdU TUNEL assay kit [24]. The mean $\pm$ SD percentages of apoptotic-like cells as determined by the TUNEL technique were 10% $\pm$ 5%, 20% $\pm$ 8%, and 22% $\pm$ 10% for 0.6, 1.2, and 2.4 μCi of ^{213}Bi-18B7/1 $\times$ 10^5 cells, respectively, with the difference between the groups treated with 0.6 and 1.2 μCi being significant ($P = .04$).

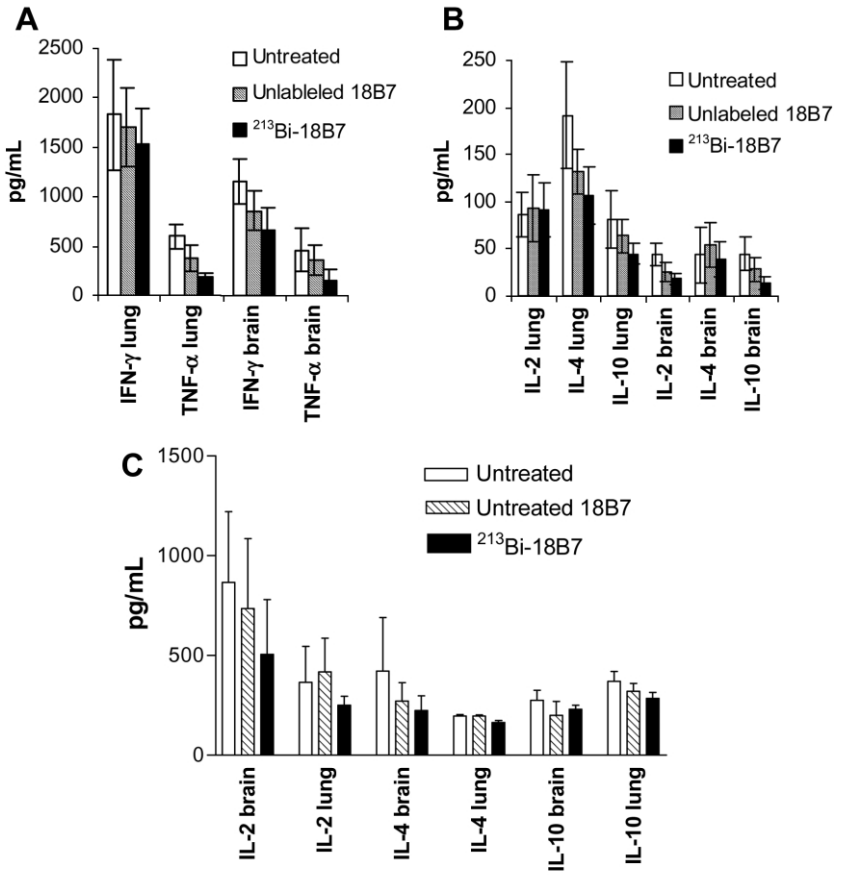

Figure 6. Levels of cytokines in the lungs and brains of AJ/Cr mice infected intravenously with *Cryptococcus neoformans* strain H99 (*A* and *B*) or 24067 *(C)*. All samples for cytokine measurement were prepared in duplicate; data shown are means ± SDs. For H99, there were statistically significant reductions ($P < .05$) in the expression of tumor necrosis factor (TNF)–α, interferon (IFN)–γ, interleukin (IL)–10 (lungs and brains), IL-4 (lungs), and IL-2 (brains) in radioimmunotherapy (RIT)–treated mice, compared with that in untreated control mice. There was a trend toward increased IL-2 expression in the lungs of RIT-treated mice ($P = .06$); also, unlabeled 18B7 monoclonal antibody caused some increase in IL-4 expression in the brains of mice ($P = .55$). For strain 24067, the reductions in IL-2, IL-4, and IL-10 expression in both the lungs and brains of RIT-treated mice, compared with those in untreated control mice, were statistically significant ($P < .02$).

Cooperation of macrophages with ^{213}Bi-labeled MAb in killing of* C. neoformans *cells. Initially, we determined whether radiolabeled MAb affected the viability of macrophages. No effect on macrophage viability was seen with doses of up to 8 μCi of ^{213}Bi-18B7 (data not shown). However, the presence of ^{213}Bi-18B7 significantly enhanced the killing of *C. neoformans* by J774.16 cells, compared with that seen with unlabeled MAb (figure 5*A*), and the process was less efficient in DMEM and mouse serum than it was in PBS (figure 5*B*).

Reduction of expression of cytokines in vivo by RIT. There was a 2-fold reduction of fungal burden in the lungs and brains of RIT-treated mice infected with strain H99 (mean ± SD, 1.5 ± 0.6 and $2.5 \pm 1.1 \times 10^5$ cfu/g of tissue, respectively), compared with that seen in control groups (mean ± SD, 3.4 ± 1.0 and $5 \pm 2.0 \times 10^5$ cfu/g of tissue, respectively) ($P = .03$). Hence, H99 strain was susceptible to killing with radiolabeled MAb in vivo, but the efficacy was lower than reported in our previous experiments with the 24067 strain [2]. RIT was associated with changes in cytokine expression in the lungs for both the H99 strain (figure 6*A* and 6*B*) and the 24067 strain (figure 6*C*). For the H99 strain, there was a significant reduction ($P < .05$) in the expression of TNF-α, INF-γ, and IL-10 (brains and lungs), IL-4 (lungs), and IL-2 (brains) in the RIT-treated mice, compared with that in the untreated control mice. There was a trend toward increased IL-2 expression in the lungs of the RIT-treated mice ($P = .06$); also, unlabeled 18B7 MAb caused some increase in IL-4 expression in the brains of the mice. For the 24067 strain, a significant reduction ($P < .02$) in the expression of IL-2, IL-4, and IL-10 in both the lungs and brains of the RIT-treated mice was detected, compared with that in the untreated control mice.

Association between RIT and fewer inflammatory cells. There were significantly fewer $CD45^+$ cells in the lungs of mice treated with unlabeled MAb or ^{213}Bi-18B7 than in the untreated

Table 2. Changes in the percentage of inflammation-related cells in the lungs of AJ/Cr mice treated with ^{213}Bi-18B7 monoclonal antibody.

	Treatment group			*P*		
Cell type	Control	Unlabeled 18B7	^{213}Bi-18B7	Unlabeled vs. control	^{213}Bi-18B7 vs. control	Unlabeled vs. ^{213}Bi-18B7
$CD45^+$	69.1 ± 4.4	52.8 ± 3.9	58.4 ± 3.1	.03	.09	.3
$CD4^+$	9.2 ± 0.8	5.4 ± 0.3	5.9 ± 0.8	.004	.02	.6
$CD8^+$	14.8 ± 2.4	8.8 ± 1.0	12.8 ± 0.7	.01	.3	.01

NOTE. Data are mean ± SD percentage of cells. *P* values were determined by Student's *t* test.

control mice (table 2). No difference in $CD45^+$ cell numbers was observed between the unlabeled MAb and ^{213}Bi-18B7 groups. Significantly fewer $CD4^+$ cells were found in the unlabeled MAb and ^{213}Bi-18B7 groups than in the untreated control mice, whereas the decrease in $CD8^+$ cells in the ^{213}Bi-18B7–treated mice did not achieve statistical significance. However, in the unlabeled 18B7–treated mice, there was a significant decrease in the number of $CD8^+$ cells.

DISCUSSION

There is a wealth of preclinical and clinical information about interactions between radiolabeled antibodies and tumor cells and about RIT-associated toxicity [3, 6, 25–28]. For example, a reduced therapeutic effect against breast cancer and lymphoma has been observed when Fc receptor activation was absent [29]. However, no comparable information is available for RIT for infectious diseases.

The *C. neoformans* MAb system permits exploration of fundamental problems in radiobiology that have not been addressed previously. Because MAb binding to *C. neoformans* is not complicated by internalization of immunoglobulin, we were able to separate direct-hit and crossfire effects by using heat-killed cells with bound ^{213}Bi-18B7 as a source of crossfire radiation. For ^{213}Bi-18B7, the mechanism of killing involved both direct-hit and crossfire effects, whereas, for ^{188}Re, killing was caused almost exclusively by crossfire effects, a finding that is consistent with the radiological properties of these isotopes. If the direct-hit effect is the major mechanism for fungal cytotoxicity with ^{213}Bi-18B7, then one would expect efficacy to be a function of affinity, because the isotope needs to be delivered in close proximity to the target cell. Consistent with this prediction, cell-killing experiments with ^{213}Bi-3E5 MAbs revealed that killing was proportional to the affinity constant.

Another mechanism that could potentially contribute to the killing of *C. neoformans* by RIT is the formation of ROS, an effect that may be catalyzed by antibodies [10]. However, ROS are unlikely to contribute significantly to fungal-cell killing, because no significant oxidant formation was observed for the dose of 0.5 μCi/1 $\times$ 10^5 cells, which was fungicidal.

The polysaccharide capsule can mediate many deleterious effects on host immune responses [30]. Previously, we showed that ^{188}Re-18B7 binding to *C. neoformans* reduced the capsule volume of H99 cells [31]. Here, we demonstrate that ^{213}Bi-8B7 has a similar effect, thus extending this observation to α-radiation. Reduction in capsule size may decrease the virulence of individual cells and, thus, contribute to the therapeutic effect of RIT. The susceptibility of different *C. neoformans* strains to RIT was clearly dependent on the ability of a given strain to bind to the radiolabeled MAb (figure 3 and table 1), with 24067 (for which the 18B7 affinity constant was the highest) being more susceptible to RIT than H99.

Environmental stresses, antibiotics, and UV light can induce apoptosis-like death in fungal cells [20–22]. Here, we show that radiation can induce dose-dependent apoptosis-like changes in *C. neoformans*. Consequently, radiation-induced apoptosis-like events may contribute to the efficacy of RIT for infectious diseases in a manner similar to the RIT effects on tumor cells [6].

Macrophages remained viable in the presence of radiolabeled MAb, but the efficacy of fungal killing by J774.16 cells was significantly enhanced by ^{213}Bi-18B7, compared with that seen with unlabeled MAb. This cooperation was impressive in view of the fact that the efficacy of *C. neoformans* killing by ^{213}Bi-18B7 alone in DMEM or in mouse serum was reduced, compared with the efficacy of killing in PBS. Serum and DMEM contain sugars, proteins, and vitamins that may attenuate the fungicidal effects of RIT. It is also possible that the binding of radiolabeled MAb to *C. neoformans* cells might be reduced in DMEM and serum.

Given that MAb-mediated protection in the setting of *C. neoformans* infection may be in part due to changes in cytokine expression [8, 11], we investigated whether RIT affected IL-2, IL-4, IL-10, TNF-α, and IFN-γ expression in RIT-treated infected mice. These cytokines are representative of the Th1- and Th2-related cytokines that are important in the effective immune response to *C. neoformans* infection [8, 32, 33]. The reduction in the organ fungal load observed in the present experiments in strain H99–infected mice was lower than that previously observed for strain 24067–infected mice [2], which is likely to be the direct consequence of decreased binding of radiolabeled 18B7 to H99 cells and decreased killing by radi-

ation. Ben-Yosef et al. [34] observed a reduction in the number of colony-forming units in the brains of mice infected systemically with *C. neoformans* and subjected to low doses of external γ-radiation. One possible explanation of the reduction in cytokine expression might be decreased inflammation in the organs as a result of decreased fungal burden. However, it is also plausible that some of the inflammatory cells were killed by crossfire radiation from the radiolabeled MAb. The reduction in cytokine expression was the same for both the H99 strain and the 24067 strain, and the percentage of change did not correlate with the change in microbial load. We note that there was no significant difference in the total number of $CD45^+$ cells in the lungs of mice treated with labeled and nonlabeled 18B7, which would argue against wholesale killing of inflammatory cells at sites of infection. However, it is conceivable that local radiation could have affected the number or location of inflammatory cells, thus resulting in an alteration in cytokine expression. In this regard, external ionizing radiation can lower $CD4^+$ and $CD8^+$ cell counts in radiation workers and in patients with cancer undergoing radiation therapy [35, 36]. Given the hypothesis that the death of *C. neoformans*–infected mice can be caused by host inflammatory damage [32], the lower percentage of $CD4^+$ cells in RIT-treated mice raises the tantalizing possibility of benefit from reduced inflammatory damage.

In summary, we identified 4 potential mechanisms by which RIT may affect the outcome of *C. neoformans* infection in treated mice: direct fungicidal effects that may follow promotion of apoptosis-like events, reduction in capsule size, enhancement of *C. neoformans* killing by macrophages, and subtle changes in the inflammatory response. In contrast, we could not implicate ROS in the fungicidal effects. Hence, the beneficial effects of RIT are probably mediated through a variety of mechanisms. The finding that affinity correlated with efficacy strongly suggests that higher-affinity MAbs would be superior reagents for use in RIT.

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