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Radioimmunotherapy Is Effective against High-Inoculum *Cryptococcus neoformans* Infection in Mice and Does Not Select for Radiation-Resistant Cryptococcal Cells▽

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We investigated the utility of radioimmunotherapy (RIT) in the treatment of experimental cryptococcal infection with high-inoculum and the possibility of RIT treatment selecting for fungal cells with radiation-resistant phenotypes. RIT reduced mortality in high-burden infections, and we found no evidence for the development of radiation-resistant cells.

In response to the need for novel treatments for infectious diseases, our laboratory has been developing a radioimmunotherapy (RIT) approach (reviewed in reference 4). *Cryptococcus neoformans*, our model organism, has well-characterized antibody reagents and animal models. We previously reported that the survival of A/JCr mice systemically infected with 10^5 *C. neoformans* cells was significantly prolonged by treatment with beta emitter 188-rhenium (^{188}Re)- or alpha emitter 213-bismuth (^{213}Bi)-labeled monoclonal antibody (MAb) 18B7, which recognizes the polysaccharide capsule of *C. neoformans* (5). Clinically, patients present at different stages of infection, some with high microbial burdens for which the efficacy of RIT is unknown. Another question is whether RIT selects for radiation-resistant fungal cells, which would interfere with follow-up RIT.

We hypothesized that ^{188}Re, which has a 16.9-h physical half-life, would be more likely than ^{213}Bi (46-min half-life) (1) to deliver radioactivity carried by MAb 18B7 (3) to 10^6 *C. neoformans* cells (strain 24067; ATCC, Manassas, VA). Our animal experiments followed the guidelines of the Albert Einstein College of Medicine Institute for Animal Studies. Groups of five A/JCr mice (NCI; Bethesda, MD) were infected i.v. with 10^6 *C. neoformans* cells and treated intraperitoneally 24 h later with 100 to 200 μCi of ^{188}Re-18B7 (30 μg MAb per mouse) or 30 μg of unlabeled 18B7. A/JCr mice were used because they are highly susceptible to i.v. infection, possibly due to a partial complement deficiency (9). Infection with 10^6 *C. neoformans* cells delivers a high inoculum that translates into a high organism burden and increased levels of glycoronoxylomannan (GXM), as would be expected in an established infection. In fact, even in infections with 10^5 cells, the levels of GXM in the blood of A/JCr mice are equal to those in patients with cryptococcosis (5).

Kaplan-Meyer plots (Fig. 1a) showed that all doses of ^{188}Re-18B7 significantly ($P < 0.05$) prolonged survival; 125 and 150 μCi were most effective, and 200 μCi was least effective. These doses should deliver radiation to any *C. neoformans* cells in the host that can be accessed by a labeled antibody. There would be 8×10^9 *C. neoformans* cells 24 h after infection with 10^6 cells; 100 μCi ^{188}Re contain 3.2×10^{11} atoms, at least 50 radioactive atoms per *C. neoformans* cell. This study with mice systemically infected with 10^6 *C. neoformans* cells demonstrates that RIT can reduce mortality even with high fungal burdens. Previously, we reported decreased fungal burdens in lungs and brains following treatment with ^{188}Re (5), where the survival rate of mice infected with 10^5 *C. neoformans* cells was the highest in the group treated with 100 μCi, while the organ fungal burden was the lowest for those treated with 200 μCi. There is no linear dose response in RIT in general (reviewed in reference 8), and with the increased infection burden the therapeutic window seems to narrow. Hematologic toxicity at the high end of the dose curve seems to outweigh the therapeutic benefit of reduction of the fungal burden by high doses (7).

A second goal was to evaluate the retention of RIT sensitivity in *C. neoformans* cells isolated from RIT-treated mice. The emergence of radiation-resistant cells would be a concern for subsequent RIT and the therapeutic outcome. To generate RIT-treated *C. neoformans* cells, A/JCr mice were infected i.v. with 5×10^4 cells and treated 24 h later with either 150 μCi ^{188}Re-18B7 or 125 μCi ^{213}Bi-18B7 or were left untreated. The surviving mice were sacrificed, and their lungs were homogenized and plated on Sabouraud's agar. Isolated colonies were grown overnight in Sabouraud's broth. To assess the radiosensitivity of the cells in vitro, *C. neoformans* cells from ATCC (CN_{naive} cells), *C. neoformans* cells recovered from untreated A/JCr mice ($CN_{passaged}$ cells), and *C. neoformans* cells recovered from mice given ^{188}Re-18B7 MAb ($CN_{Re\ RIT}$ cells) or ^{213}Bi-18B7 MAb ($CN_{Bi\ RIT}$ cells) were treated with ^{188}Re-18B7 or ^{213}Bi-18B7 MAb as previously described (2). Naive,

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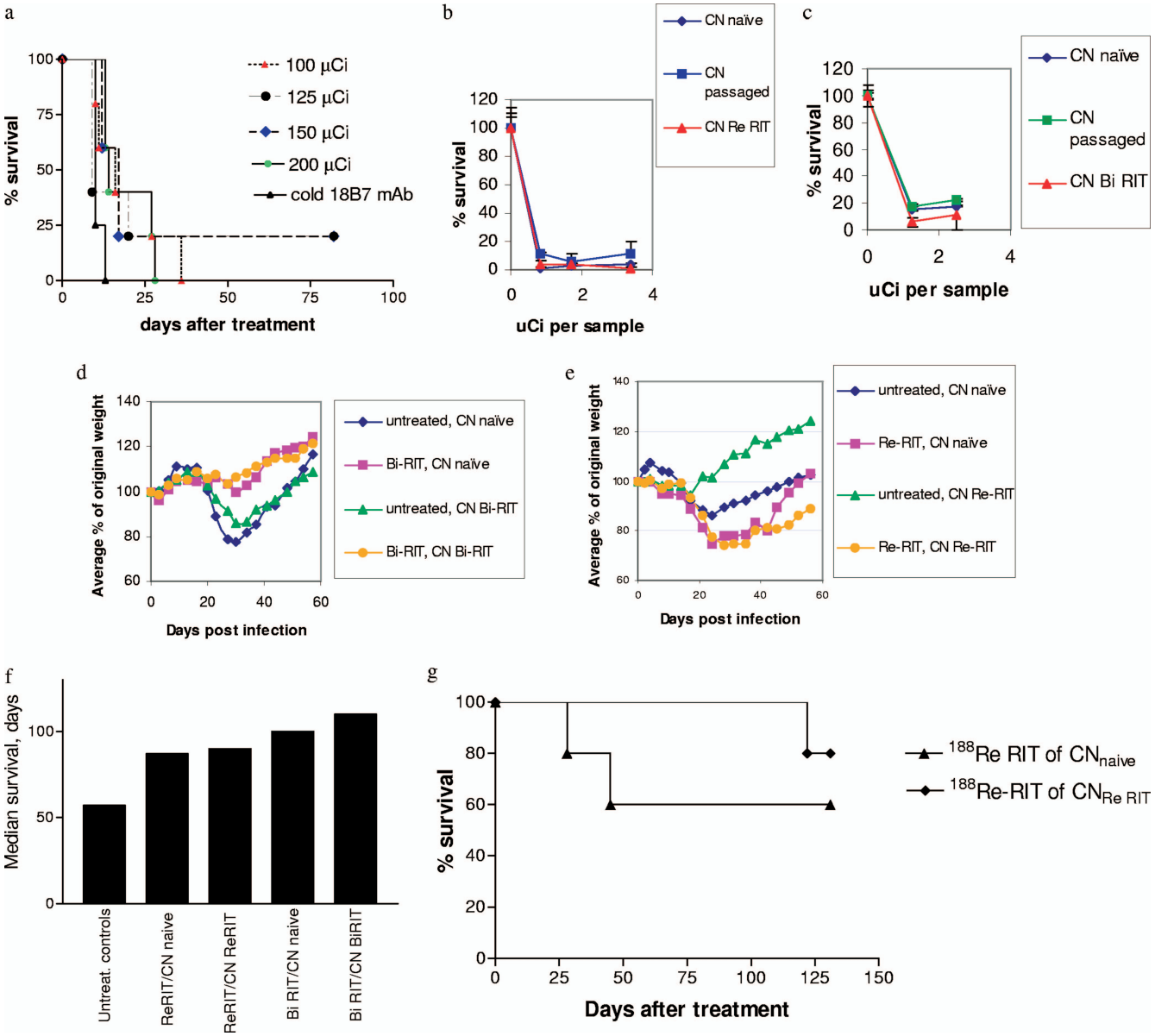

FIG. 1. RIT of *C. neoformans* in vivo and in vitro. (a) Survival percentages for A/JCr mice infected i.v. with 10^6 *C. neoformans* cells and treated 24 h later with 100 to 200 µCi ^{188}Re-18B7 MAb. Control mice were given matching amounts of unlabeled (cold) 18B7 MAb. (b) In vitro killing of *C. neoformans* with ^{188}Re-18B7 MAb. Each sample contained 10^5 fungal cells. (c) In vitro killing of *C. neoformans* with ^{213}Bi-18B7 MAb. Each sample contained 10^5 fungal cells. (d) Average percentages of body weight change in ^{213}Bi-18B7-treated and control mice. (e) Average percentages of body weight change in ^{188}Re-18B7-treated and control mice. (f) Median survival in days of A/JCr mice infected i.v. with 5×10^4 *C. neoformans* cells and treated 24 h later with 150 µCi ^{188}Re-18B7 or 125 µCi ^{213}Bi-18B7 MAb. Untreat., untreated; ReRIT/CN naive, mice infected with CN_{naive} cells and treated with ^{188}Re-18B7; Bi RIT/CN naive, mice infected with CN_{naive} cells and treated with ^{213}Bi-18B7; ReRIT/CN ReRIT, mice infected with $CN_{Re\ RIT}$ cells and treated with ^{188}Re-18B7; Bi RIT/CN BiRIT, mice infected with $CN_{Bi\ RIT}$ cells and treated with ^{213}Bi-18B7. (g) Kaplan-Meyer curves showing survival of A/JCr mice infected i.v. with 5×10^4 *C. neoformans* cells and treated 24 h later with 150 µCi ^{188}Re-18B7 MAb.

passaged, or RIT-pretreated cells were equally radiosensitive to both ^{188}Re and ^{213}Bi (Fig. 1b and c).

To evaluate the possibility that RIT might select for *C. neoformans* cells resistant to radiation in vivo, we infected A/JCr mice with $CN_{Re\ RIT}$, $CN_{Bi\ RIT}$, and CN_{naive} cells. Infected mice were treated with 150 µCi ^{188}Re-18B7 or 125 µCi ^{213}Bi-18B7 24 h post-i.v. infection and then monitored for survival and weight loss. We detected no differences in weight loss for mice infected with $CN_{naïve}$ cells and mice infected with $CN_{Re\ RIT}$ or $CN_{Bi\ RIT}$ cells. All groups lost weight after infection (Fig. 1d and e); however, mice receiving RIT with ^{213}Bi-18B7 lost significantly less weight at the nadir (27 to 30 days) than untreated controls ($P < 0.007$ by Student's t test) (Fig. 1d). By contrast, the trend for groups treated with ^{188}Re-18B7 was to lose more weight than untreated groups ($P = 0.06$) (Fig. 1e). RIT with ^{188}Re-18B7 was more radiotoxic in mice with

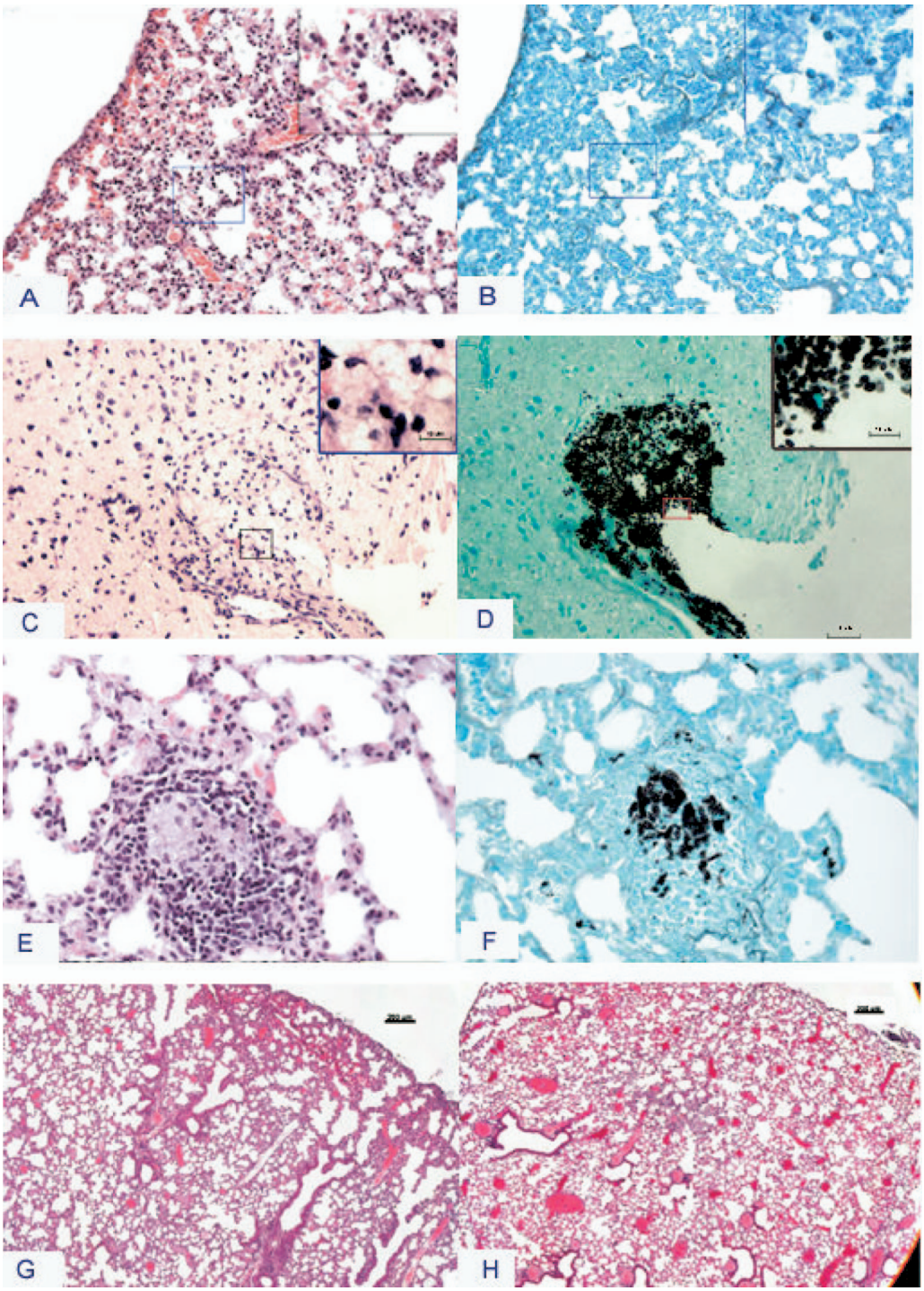

FIG. 2. Histology of brains and lungs from A/JCr mice infected i.v. with 5×10^4 *C. neoformans* cells and treated after 24 h with 125 μCi ^{213}Bi-18B7 MAb. Mice were sacrificed 3 months posttreatment. (a, c, e, g, and h) Hematoxylin and eosin staining. (b, d, and f) GMS staining. (a and b) Lung from a ^{213}Bi-18B7-treated CN_{naive} mouse, showing scattered alveolar macrophages with GMS-positive material within the cytoplasm (×200 magnification). The insert represents expansion of the boxed region. (c and d) Brain from a ^{213}Bi-18B7-treated $CN_{Bi\ RIT}$ mouse showing lymphohistiocytic meningitis at the base of the brain, with intralesional cryptococci (×200 magnification). The insert represents expansion of the boxed region. (e and f) Lungs from the same mouse as in panels c and d, showing a focal granuloma with central foamy macrophages which are encircled by lymphocytes. Macrophages contain GMS-positive organisms (×400 magnification). (g and h) Overview of the lungs (×25 magnification). (g) Lung from mouse infected with CN_{naive} and treated with ^{213}Bi-18B7. (h) Lung from mouse infected with $CN_{Bi\ RIT}$ and treated with ^{213}Bi-18B7. All magnifications are original.

chronic *C. neoformans* lung infection than RIT with ^{213}Bi-18B7 (7); the longer range of ^{188}Re emissions in tissue may damage healthy tissues. Lethality in mice infected with $CN_{Re\ RIT}$ or $CN_{Bi\ RIT}$ cells was the same as in mice infected with $CN_{naïve}$ cells ($P > 0.05$) (Fig. 1f). The survival of mice treated with ^{213}Bi-18B7 MAb was longer ($P = 0.04$) than of those treated with ^{188}Re-18B7 (Fig. 1g), probably due to the higher killing power of alpha particles from ^{213}Bi than of electrons from ^{188}Re.

At 130 days postinfection, the lungs and brains from selected mice from each group were plated for CFU or analyzed histologically for signs of inflammation, possible radiation scarring (by using hematoxylin and eosin stain), and the presence of *C. neoformans* cells (by using Gomori methenamine-silver nitrate stain [GMS]). No striking difference between the groups was evident. The pathology in these chronically infected mice was generally focal and circumscribed, consisting of areas of lymphocytic and histiocytic infiltrates in areas containing cryptococcal cells (Fig. 2). Organ cultures from some mice from each treatment group had no CFU, consistent with the clearance of infection in both the brain and lung. Radiation fibrosis in the lungs was nonexistent (Fig. 2), consistent with previous observations (7).

Treatment of *C. neoformans* with particulate radiation leads to the loss of clonogenicity (6, 2), which would explain the absence of radiation-resistant phenotypes after RIT. The residual cells which replicate after RIT most likely were protected from radiolabeled antibodies by a biofilm, an abscess, or a host cell. Like other antifungal therapies, RIT reduces the cryptococcal burden but does not eradicate infection. The efficacy of RIT might be enhanced by combining it with antifungal drugs or by repeated fractionated treatments. RIT provides a novel approach to antifungal therapy, potentially applicable to a wide spectrum of human mycoses.

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