Radioimmunotherapy is effective against high inoculum *Cryptococcus neoformans* infection in mice and does not select for radiation-resistant cryptococcal cells

*Running title: RIT of high inoculum and radio-treated cells*

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ABSTRACT

We investigated the utility of radioimmunotherapy (RIT) in 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 infection and we found no evidence for the development of radiation resistant cells.

Key words: *C. neoformans*, radioimmunotherapy, high inoculum infection, radiation-resistant phenotypes, 213-Bismuth, 188-Rhenium
In response to the need for novel treatments for infectious diseases, our laboratory has been developing radioimmunotherapy (RIT) approach (reviewed in 4). *Cryptococcus neoformans* (CN), our model organism, has well-characterized antibody reagents and animal models. We previously reported that treatment, using beta-emitter $^{188}$Rhenium ($^{188}$Re)- or alpha-emitter $^{213}$Bismuth ($^{213}$Bi)-labeled monoclonal antibody (mAb) 18B7 recognizing CN’s polysaccharide capsule, of AJC/r mice systemically infected with $10^5$ CN cells significantly prolonged survival of these mice (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, with a 16.9 hour physical half-life, would be more likely than $^{213}$Bi (46 min half-life) to deliver radioactivity carried by mAb 18B7 (2) to $10^6$ CN cells (strain 24067, ATCC (Manassas, VA)). Animal experiments followed guidelines of Albert Einstein College of Medicine Institute for Animal Studies. Groups of five AJC/r mice (NCI, Bethesda, MD) were infected IV with $10^6$ CN cells and treated IP 24 hr later with 100-200 $\mu$Ci $^{188}$Re-18B7 (30 $\mu$g mAb per mouse), or 30 $\mu$g unlabeled 18B7. A/JCr mice were used because they are highly susceptible to IV infection, possibly due to partial complement deficiency (9). Infection with $10^6$ CN cells delivers high inoculum that would translate into high organism burden and increased levels of GXM, as would be expected in established infection. In fact, even in infection with $10^5$ cells levels of GXM in AJ/Cr mice blood are equal to those in patients with cryptococcosis (5).
Kaplan-Meyer plots (Fig. 1a) show 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 CN in the host that can be accessed by labeled antibody: there would be $8 \times 10^9$ CN cells 24 h after $10^6$ infection; 100 µCi $^{188}$Re contain $3.2 \times 10^{11}$ atoms, at least 50 radioactive atoms per CN cell. This study in mice systemically infected with $10^6$ CN cells demonstrates that RIT can reduce mortality even in high fungal burdens. Previously we reported decreased fungal burden in lungs and brains following treatment with $^{188}$Re (5), where the survival of mice infected with $10^5$ CN cells was the highest in the group treated with 100 µCi while the organ fungal burden was the lowest for 200 µCi. There is no linear dose response in RIT in general (reviewed in 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 fungal burden reduction by high doses (7).

A second goal was to evaluate retention of RIT sensitivity in CN cells isolated from RIT-treated mice. The emergence of radiation-resistant cells would be a concern for subsequent RIT, and therapeutic outcome. To generate RIT-treated CN cells, AJ/Cr mice were infected IV with $5 \times 10^4$ cells and 24 hrs later treated with either 150 µCi $^{188}$Re-18B7 or 125 µCi $^{213}$Bi-18B7 or left untreated. The surviving mice were sacrificed, their lungs homogenized and plated on SAB agar; isolated colonies were grown overnight in SAB broth. To assess radiosensitivity of the cells in vitro, cells from ATCC (CN$_{naive}$), recovered from untreated AJ/Cr mice (CN$_{passaged}$) and recovered from mice given $^{188}$Re-18B7 mAb (CN$_{Re\, RIT}$) or $^{213}$Bi -18B7 mAb (CN$_{Bi\, RIT}$)
were treated with $^{188}$Re- or $^{213}$Bi-18B7 mAb as in (2). Naive, passaged or RIT pre-treated cells were equally radiosensitive to both $^{188}$Re and $^{213}$Bi (Fig. 1b,c).

To evaluate the possibility that RIT might select for CN cells resistant to radiation in vivo, we infected AJ/Cr mice with CN_{Re-RIT}, CN_{Bi-RIT} and CN_{naive}. Infected mice were treated with 150 µCi $^{188}$Re-18B7 or 125 µCi $^{213}$Bi-18B7 24 hrs post IV infection, then monitored for survival and weight loss. We detected no differences in weight loss in mice infected with CN_{naive} compared to mice infected with CN_{Re-RIT} or CN_{Bi-RIT}. All groups lost weight after infection (Fig. 1d, e), however, mice receiving RIT with $^{213}$Bi-18B7 lost significantly less weight at nadir (27-30 days) than untreated controls (P<0.007 by Student’s t test) (Fig. 1d). By contrast, groups treated with $^{188}$Re-18B7, showed trend in losing more weight than untreated groups (P=0.06) (Fig. 1e). RIT with $^{188}$Re-18B7 was more radiotoxic in mice with chronic CN 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} was the same as in mice infected with CN_{naive} (P>0.05) (Fig. 1f). Survival of mice treated with $^{213}$Bi-18B7 mAb was longer (P=0.04) than with $^{188}$Re-18B7 (Fig. 1g), probably due to the higher killing power of alpha particles from $^{213}$Bi, compared to electrons from $^{188}$Re.

At 130 days post infection, the lungs and the brains from selected mice from each group were plated for CFU’s or analyzed histologically for signs of inflammation, possible radiation scarring (H&E staining) and presence of CN cells (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/histiocytic infiltrates in areas containing cryptococcal cells (Fig. 2). Organ
cultures from some mice from each treatment group had no CFUs, consistent with clearance of
infection in both brain or lung. Radiation fibrosis in the lungs was non-existent (Fig. 2),
consistent with previous observations (7).

Treatment of CN with particulate radiation leads to 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 cryptococcal burden but does not eradicate infection. The efficacy of RIT might be enhanced by combination with anti-fungal 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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FIGURE LEGENDS

Fig. 1. RIT of CN cells in vivo and in vitro: a) survival of AJ/Cr mice infected IV with 10^6
CN and treated 24 hrs later with 100 - 200 μCi ^{188}Re-18B7 mAb. Control mice were given
matching amounts of unlabeled ("cold") 18B7 mAb; b) in vitro killing of CN cells with ^{188}Re-
18B7 mAb. Each sample contained 10^5 fungal cells; c) in vitro killing of CN cells with ^{213}Bi-
18B7 mAb. Each sample contained 10^5 fungal cells; d) average percentage in body weight
change in ^{213}Bi-18B7-treated and control mice; e) average percentage in body weight change in
^{188}Re-18B7-treated and control mice; f) median survival of AJ/Cr mice infected IV with 5x10^4
CN and treated 24 hrs later with 150 μCi ^{188}Re-18B7 or 125 μCi ^{213}Bi-18B7 mAb; g) Kaplan-
Meyer curves showing survival of AJ/Cr mice infected IV with 5 x 10^4 CN and treated 24 hrs
later with 150 μCi ^{188}Re-18B7 mAb. CN naive - cells from ATCC; CN passaged - cells recovered
from untreated AJ/Cr mice; CN Re RIT - cells recovered from mice treated with ^{188}Re-18B7
mAb; CN\textsubscript{Bi RIT} - cells recovered from mice treated with \textsuperscript{213}Bi-18B7 mAb; Re RIT/ CN\textsubscript{naive} - mice infected with CN\textsubscript{naive} and treated with \textsuperscript{188}Re-18B7; Bi RIT/ CN\textsubscript{naive} - mice infected with CN\textsubscript{naive} and treated with \textsuperscript{213}Bi-18B7; Re RIT/CN\textsubscript{Re RIT} - mice infected with CN\textsubscript{Re RIT} and treated with \textsuperscript{188}Re-18B7; Bi RIT/CN\textsubscript{Bi RIT} - mice infected with CN\textsubscript{Bi RIT} and treated with \textsuperscript{213}Bi-18B7.

Fig. 2 Histology of brains and lungs from AJ/Cr mice infected IV with $5 \times 10^4$ CN and treated after 24 hrs with 125 $\mu$Ci \textsuperscript{213}Bi-18B7 mAb. Mice were sacrificed 3 months post-treatment: a), c), e), g), and h) - H&E staining; b), d) and f) - GMS staining. a) and b) lung from a Bi RIT/ CN\textsubscript{naive} mouse, showing scattered alveolar macrophages with GMS positive material within the cytoplasm (200X magnification); insert represents expansion of the boxed region; c) and d) - brain from Bi RIT/ CN\textsubscript{Bi-RIT} mouse showing lymphohistiocytic meningitis at the base of the brain, with intrallesional cryptococci (200X magnification); insert represents expansion of the boxed region; e) and f) - lungs from the same mouse as in c) and d), showing a focal granuloma with central foamy macrophages which are encircled by lymphocytes. Macrophages contain GMS positive organisms (400X magnification); g) and h) – 25X magnification overview of the lungs: g) - lung from Bi-RIT/ CN\textsubscript{naive} mouse (infected with CN\textsubscript{naive} and treated with \textsuperscript{213}Bi-18B7); h) - lung from Bi-RIT/CN\textsubscript{Bi-RIT} mouse (infected with CN\textsubscript{Bi RIT} and treated with \textsuperscript{213}Bi-18B7).
Fig. 1a-e
Fig. 1f, g