Treatment of early and established Cryptococcus neoformans infection with radiolabeled antibodies in immunocompetent mice

Running title: RIT of C. neoformans in immunocompetent mice

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We investigated the utility of radioimmunotherapy in early and established cryptococcal infection in immunocompetent mice. RIT with $^{213}$Bi-18B7 antibody completely eliminated fungus from mice lungs and brains for early infection, while $^{188}$Re-18B7 significantly reduced CFUs in the lungs or both lungs and brains during early and established infection, respectively. The results point to the independence of RIT on the immune status of the host which is encouraging for translation of this strategy into the clinic.

**Key words:** *C. neoformans*, radioimmunotherapy, established infection, C57BL6 mice, 213-Bismuth, 188-Rhenium
The inability of immunocompromised individuals to mount an effective immune response to fungal infections reduces the efficacy of standard antifungal therapies. For example, *Cryptococcus neoformans* (CN) infections cause life threatening meningoencephalitis primarily in immunocompromised patients, causing more deaths than tuberculosis among AIDS patients in the developing world (9). Our laboratory is developing a radioimmunotherapy (RIT) approach as a novel treatments for infectious diseases (7). The RIT relies on the antigen-binding characteristics of antibodies to deliver cytotoxic radiation to target cells (12). Radiolabeled monoclonal antibodies (mAbs) Zevalin® and Bexxar® are FDA-approved for untreated, refractory and recurrent lymphomas. Several years ago we demonstrated the potential efficacy of RIT against infectious diseases by showing prolonged survival in mice systemically infected with CN and treated post-infection with radiolabeled 18B7 mAb to CN polysaccharide capsule (8). Subsequently, we applied RIT against bacteria and HIV (7). This approach showed little toxicity (6), and work has begun to uncover the radiobiological and immune mechanisms of RIT (5, 1). We previously tested the ability of $^{213}$Bi-18B7 to kill more virulent H99 strain in A/JCr mice, and found that it significantly reduced the fungal burden in both lungs and brains 24 hours post treatment (5). Very recently, we demonstrated that RIT was more efficient than standard antifungal therapy in treating systemic CN infection in mice (3).

Until now, all studies of RIT of systemic CN infection were performed in A/JCr mice because they are highly susceptible to IV infection, possibly due to partial complement deficiency (10) and RIT was also always administered 24 hrs post-infection with CN. However, this reliance on one model led to concerns about its general applicability in other hosts and whether RIT
would retain efficacy in settings of more established infection. Since RIT relies primarily on
the killing of the microbes by cytocidal radiation - it would be important to demonstrate its
independence of the host immune status. In addition, demonstrating the efficacy of RIT
towards the established infections characterized by the high fungal load would be beneficial for
its future applications in clinical practice. Experimental and natural infection may not be
comparable but experimental infection is the basis for all available models and 48 hr is
sufficient for an organism that replicates every 2-4 hr in vivo. In this study we aimed to
investigate whether: 1) CN systemic infection in immunocompetent C57BL6 mice is amenable
to RIT at 24 hrs; 2) established CN systemic infection at 48 hrs could be treated with RIT in
the same mouse strain.

We hypothesized that at 24 hrs after infection both alpha-emitter 213-Bismuth (\(^{213}\)Bi, 46 min
physical half-life) and beta-emitter 188-Rhenium (\(^{188}\)Re, 16.9 hr half-life) should be capable of
killing fungal cells in infected C57BL6 mice when carried to the sites of the infection by the
mAb 18B7. We also hypothesized that \(^{188}\)Re which has more radioactive atoms per unit of
activity than \(^{213}\)Bi will be a more effective choice of a radionuclide for treatment of 48 hr
infection which is characterized by higher microbial burden.

CN strain 24067 was obtained from ATCC (Manassas, VA) and the cells grown as in (3).
Animal experiments followed guidelines of Albert Einstein College of Medicine Institute for
Animal Studies. Groups of 5-8 C57BL6 female 8 to 10 weeks old mice (Jackson Laboratories)
were infected IV via tail vein with \(10^6\) CN cells and were left untreated or treated IP with: 100
\(\mu\text{Ci} \, ^{213}\text{Bi}-18B7\) 24 hr post-infection; 100 \(\mu\text{Ci} \, ^{188}\text{Re}-18\text{B7}\) 24 hr post-infection; 100 \(\mu\text{Ci} \, ^{188}\text{Re}-\)}
18B7 48 hrs post-infection. Radiolabeling of 18B7 mAb with $^{213}$Bi or with $^{188}$Re was performed as in (5). The total amount of 18B7 mAb per mouse was 30 µg which has been shown to have no effect on infection burden in AJ/Cr mice (5, 8). Mice were monitored for their survival for 75 days, humanely sacrificed, their brains and lungs removed, divided in half, and one half was stained and analyzed histologically for signs of inflammation and possible radiation scarring (hematoxylin and eosin (H&E)) and presence of CN cells (Gomori Methenamine-Silver Nitrate stain (GMS)). The remaining tissue was disrupted, diluted, and plated for CFUs. Differences in CFUs between the groups were analyzed by Student’s t-test for unpaired data. P values of <0.05 were considered significant.

None of the mice in the study including untreated infected controls died during the 75 days of observation pointing to the development of an indolent chronic infection. There was approximately equal microbial burden in the lungs and the brains of the untreated mice (Fig. 1a) that was confirmed by the GMS staining (Fig. 1b). These data are in concordance with the previous studies on CN infection of C57BL6 mice (11, 14) and in contrast with the data in AJ/Cr mice in which the fungal load in the lungs is usually several orders of magnitude higher than in the brain (8). Treatment of mice with $^{188}$Re-18B7 mAb 24 hr post-infection produced one log reduction in the CFUs in lungs (P=0.04) and none – in the brains (P=0.07) (Fig. 1a). Administration of $^{213}$Bi-18B7 24 hr post-infection completely eliminated fungal cells from both the lungs and the brains (the detection limit of plating assay was 50 CFUs) (Fig. 1a) which was confirmed by GMS staining (Fig. 1e). We observed the same elimination of the fungal burden from the lungs and brains of AJ/Cr mice infected with $10^5$ CN cells and treated with $^{213}$Bi-18B7 (3). For established infection at 48 hrs $^{188}$Re-18B7 mAb was at least as effective in
decreasing CFUs in the lungs (P=0.03) as at 24 hr and also significantly reduced the fungal burden in the brains (P=0.02) (Fig. 1a). The latter fact might reflect the increased permeability of the blood-brain-barrier with the onset of infection which facilitates the influx of the radiolabeled antibody into the brain. According to Shi et al (13), in an IV mouse model, at 24 hrs there is little penetration by CN through the brain microvasculature, with only 8% of the cells in the brain parenchyma, and the rest still in the capillaries. Therefore, at 24 hours, most of the cells would be accessible to the radiolabeled mAbs and are either completely eliminated by $^{213}$Bi-18B7 which prevents any further penetration into the brain, or are only partially eliminated by less powerful $^{188}$Re-18B7 with the remaining cells continue to penetrate into the brain. If the treatment with $^{188}$Re-18B7 is administered at 48 hrs post infection when the BBB became more permeable for the mAb - $^{188}$Re-18B7 can “chase” CN directly in the brain which results in decreasing CFUs in this organ (Fig. 1). Thus, the decrease is brain CFUs post RIT can be attributed to its direct antifungal effects. The pathology of the lungs and brains of $^{188}$Re-18B7 mAb-treated mice showed focal nature of the remaining fungal load (Fig. 1c,d). The ability of a single injection of $^{188}$Re-labeled mAb to significantly decrease microbial burden in established infection is encouraging taking into consideration that: 1) there are at least 3 orders of magnitude more fungal cells in the organs at 48 hrs post-infection in comparison with 24 hr; 2) multi-day courses of antifungals such as amphotericin B are required to achieve the same results (4). It might be possible that repeat administrations of $^{213}$Bi-18B7 mAb would result in successful treatment of an established infection. This is the 1st study where the CFUs in brain and lungs were compared in the long term survivors between $^{213}$Bi- and $^{188}$Re – labeled mAbs. While the two isotopes are similar in terms of prolonging mouse survival (8), they are different in regard to the mechanism of CN killing. $^{213}$Bi-18B7 is
extremely lethal to CN with only one or two “hits” needed for killing a cell leading to a loss of metabolic activity in treated cells, whereas $^{188}$Re has to deliver hundreds of “hits” needed for killing (1). Thus, it is possible that $^{188}$Re-18B7 is decreasing the CFUs enough to allow survival, but not completely eliminating the organisms. Lastly, analysis of the H&E stained tissues demonstrated no evidence of radiation fibrosis in the lungs and the brains (not shown), consistent with previous observations (2, 6).

In conclusion, RIT was effective against early and established infection in immunocompetent C57BL6 mice. Previously the efficacy of RIT was shown in complement deficient AJ/Cr mice. The results point to the independence of RIT on the immune status of the host which is encouraging for translation of this strategy into the clinic for treatment of cancer and organ transplant patients with opportunistic infections.

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FIGURE LEGENDS
Fig. 1. RIT of C57BL6 mice infected IV with $10^6$ CN cells: a) CFUs in the brains and the lungs of RIT-treated and control mice. Mice were treated IP with either: 100 µCi $^{213}$Bi-18B7
24 hr post-infection; 100 µCi $^{188}$Re-18B7 24 hr post-infection; 100 µCi $^{188}$Re-18B7 48 hrs post-infection; or left untreated and sacrificed 75 days post-treatment. Detection limit of the method was 50 CFUs. No CFUs were found in the brains and lungs of mice treated with 100 µCi $^{213}$Bi-18B7 which are presented in the graph as 40 CFUs/organ. The asterisks show the groups in which the CFUs were significantly different from the untreated controls; b) - e) – histology of the brains (left panels) and lungs (right panels) of RIT-treated and control mice. Organs were stained with GMS. CN cells stained with GMS appear black on the images. b) untreated mouse; c) mouse treated with 100 µCi $^{188}$Re-18B7 24 hr post-infection; d) mouse treated with 100 µCi $^{188}$Re-18B7 48 hrs post-infection; e) mouse treated with 100 µCi $^{213}$Bi-18B7 24 hr post-infection. The size bar is 100 µm.

REFERENCES


a) CFUs/organ

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<tr>
<td>¹⁸⁸Re-18B7, 24 hrs</td>
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<td>¹⁸⁸Re-18B7, 48 hrs</td>
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b) Images showing bacterial growth in lungs and brains.

c) Close-up images highlighting bacterial colonies.
Fig. 1