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Effects of radiation type and delivery mode on a radioresistant eukaryote *Cryptococcus neoformans*

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Abstract

Introduction—Most research on radioresistant fungi, particularly on human pathogens such as *Cryptococcus neoformans*, involves sparsely-ionizing radiation. Consequently, fungal responses to densely-ionizing radiation, which can be harnessed to treat life-threatening fungal infections, remain incompletely understood.

Methods—We addressed this issue by quantifying and comparing the effects of densely-ionizing α -particles (delivered either by external beam or by ^{213}Bi -labeled monoclonal antibodies), and sparsely-ionizing ^{137}Cs γ -rays, on *Cryptococcus neoformans*.

Results—The best-fit linear-quadratic parameters for clonogenic survival were the following: $\alpha=0.24\times10^{-2}\text{ Gy}^{-1}$ for γ -rays and $1.07\times10^{-2}\text{ Gy}^{-1}$ for external-beam α -particles, and $\beta=1.44\times10^{-5}\text{ Gy}^{-2}$ for both radiation types. Fungal cell killing by radiolabeled antibodies was consistent with predictions based on the α -particle dose to the cell nucleus and the linear-quadratic parameters for external-beam α -particles. The estimated RBE (for α -particles vs γ -rays) at low doses was 4.47 for the initial portion of the α -particle track, and 7.66 for the Bragg peak. Non-radiological antibody effects accounted for up to 23% of cell death.

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Conclusions—These results quantify the degree of *C. neoformans* resistance to densely-ionizing radiations, and show how this resistance can be overcome with fungus-specific radiolabeled antibodies.

Keywords

Cryptococcus neoformans; radiation; alpha particles; radiolabeled antibodies

1. Introduction

Radioresistant organisms can survive large doses of ionizing radiation (e.g. hundreds or thousands of Gy) without losing reproductive potential [1-4]. Radioresistance is probably an evolutionary by-product of other properties (e.g. resistance to desiccation and/or to other genotoxic agents), which are important for the organism's survival in its natural environment [5-7]. Consequently, different organisms may evolve resistance to radiation through different mechanisms, and even seemingly subtle modulations of evolutionarily ancient pathways (e.g. DNA repair machinery) may substantially enhance radioresistance [4, 8, 9].

Many fungi tolerate large radiation doses not only under laboratory conditions, but also survive (or even gain a competitive advantage) in environments heavily contaminated by radioisotopes [10-14]. Overcoming the radioresistance of pathogenic fungi is important for medical purposes, and fungus-specific radioimmunotherapy (RIT) shows considerable promise in accomplishing this. For example, it resulted in successful eradication of fungal pathogens *in vitro* and *in vivo* [15-17].

So far, most research on radioresistant fungi, particularly on human pathogens, has involved sparsely ionizing radiation (e.g. γ -rays). Here we employed *Cryptococcus neoformans* as a model organism. This fungus is an important human pathogen, especially in immunocompromised individuals affected by human immunodeficiency virus (HIV) [18, 19]. It is a particularly interesting model system because it is highly resistant to sparsely-ionizing radiation from external sources, but is susceptible to densely-ionizing radiation delivered by radiolabeled monoclonal antibodies specific to fungal antigens *in vitro* and *in vivo* [15, 17]. To elucidate and quantify the effects of radiation type and delivery mode on clonogenic survival of *C. neoformans*, we compared the effects of densely-ionizing particles (^4He ions delivered either by external beam, which we call external-beam α -particles for convenience, or α -particles produced by ^{213}Bi radiolabeled antibodies) and sparsely-ionizing ^{137}Cs γ -rays. Our goals were: (1) estimate α -particle doses to the cell nucleus (and to the cell body) from radiolabeled antibodies; (2) using these estimates, clarify from a dosimetric perspective how radiolabeled antibodies can produce sufficient radiation doses to kill radioresistant *C. neoformans* cells [15, 17]; (3) estimate the linear-quadratic (LQ) parameters for *C. neoformans* cell survival after exposure to γ -rays and to external-beam α -particles and determine to what extent the radioresistance of *C. neoformans* applies to densely ionizing radiation; (4) estimate α -particle RBE for *C. neoformans*; ; (5) compare the cytotoxic effectiveness (per unit of α -particle dose) of radiolabeled antibodies with the effectiveness of external-beam α -particles; and (6) assess how the responses of *C.*

neoformans to different radiation types conform to patterns observed in mammalian cell radiobiology.

2. Materials and methods

2.1. *C. neoformans* growth and size measurements

Cryptococcus neoformans (strain 24067) was obtained from ATCC and maintained on Sabouraud (SAB) agar plates. For all experiments, the cells were grown for two days in SAB media at 28°C, shaking at 150 RPM in an orbital shaker, then sub-cultured at 1/1000, to a density of approximately 5×10^5 cells/mL, into minimal medium (29.4 mM KH_2PO_4 , 10 mM MgSO_4 , 13 mM glycine, 15 mM D-glucose, 3 μM thiamine) [20]. They were then grown for 5 days, at 28°C and 150 RPM, to insure that the cells were in stationary phase. The sizes of the cells and the capsules were measured by photomicroscopy using India ink to visualize the boundary of the capsules. Images were taken using an Olympus AX70 microscope, using Q capture software. Fifteen cell and capsule diameters were measured using Adobe Photoshop. Average values are listed in Table 1, and the standard errors were 3.0-3.4% of the means.

2.2. External γ -ray and α -particle irradiation

Exposure to ^{137}Cs γ -rays was performed on two separate occasions using the Shepherd Mark I irradiator at Albert Einstein College of Medicine, at a dose rate of 10.76 Gy/min. The maximum dose was 320 Gy. The cells were cultured as described above, washed with PBS twice at 6,000 RPM for 5 minutes, adjusted to 7×10^6 cells/mL, and placed in FACS tubes for exposure.

External α -particle exposure was performed on two separate occasions at the Radiological Accelerator Research Facility (RARAF) of Columbia University, Nevis Laboratories, Irvington, NY. The cells were cultured as described above, washed twice with phosphate buffered saline (PBS), pH= 5.7 at 4,000 RPM for 5 minutes, and adjusted to 6×10^8 cells/mL. A small volume (18 μL) of this cell suspension was placed on a 6 μm thick Mylar film epoxied to the bottom of a steel ring [21, 22]. A 22 $\times$ 22 mm glass coverslip was placed over the sample to make the depth of the cell suspension uniform. The ring was covered with a wet paper towel square to delay evaporation of the sample, and the entire ring was covered with Parafilm. Irradiation by ^4He ions (here called external-beam α -particles) with initial energy of 9 MeV when exiting the RARAF accelerator, occurred through the Mylar film, penetrating the cell suspension in the vertical direction from the bottom up (Fig. 1). The particle energy when exiting the Mylar and entering the cell suspension was 7.14 MeV, and the linear energy transfer (LET) was 72 keV/ μm .

The α -particle dose rate varied between 3 and 26 Gy/min, and total exposure time was 20 minutes for any sample. The maximum dose was 150 Gy. Relatively homogeneous dose delivery to the cells was provided because the α -particle LET variation was small in the bottom portion of the liquid layer where *C. neoformans* cells have settled (<1% over 8 μm , and <3% over 12 μm). After irradiation, the Parafilm and paper towel square were removed from each sample and 0.5 mL of PBS was added to each ring, to “presoak” the samples and

assist in the detachment of cells from the Mylar. The samples were then pipetted up by in 0.5 mL of PBS, the Mylar rings were washed with another 0.5 mL of PBS, and then samples were placed into FACS tubes before being plated to enumerate the colony forming units (CFUs) as described below.

2.3. Radio-labeling of 18B7 antibody to *C. neoformans* polysaccharide capsule with ^{213}Bi

The 18B7 antibody, a kind gift from Dr. A. Casadevall at the Albert Einstein College of Medicine, binds to the polysaccharide capsule of *C. neoformans* [20, 23]. Frozen 18B7 antibody stock was first thawed and then transferred into a carbonate conjugation buffer by washing 4 times with 1.5 mL of conjugation buffer by centrifugation in a Centricon tube at 4°C for 20 minutes. A stock solution of the bifunctional chelating agent CHXA” (N-[2-amino-3-(p-isothio-cyanatophenyl) propyl]-trans-cyclohexane-1,2-diamine-N, N', N", N"', N""-pentaacetic acid) (Macrocyclics, Dallas, TX) was prepared at 2 mg/mL in carbonate buffer. The antibody was incubated with a 2-fold molar excess of CHXA” at room temperature overnight. The antibody was washed six times in a Centricon tube with 1.5 mL of 0.15 M ammonium acetate buffer (NH_4OAc) at 5,000 RPM at 4°C to remove excess CHXA” and transfer it to the ammonium acetate buffer. The conjugated antibody was stored at –20°C. 18B7 has a high binding affinity and approximately 1.1×10^6 binding sites per cell for *C. neoformans* strain 24067 [24].

The 18B7 antibody conjugated with CHXA” was placed at 37°C. ^{213}Bi was eluted from an ^{225}Ac generator using 600 μL of 0.15 M hydroiodic acid (HI). The pH of the ^{213}Bi solution was adjusted to pH 5 using 80 μL of 2.5 M NH_4OAc , then 200 μL of the resulting solution was added to 18B7-CHXA” and mixed 2-3 times. The mixture was incubated at 37°C for 6 minutes, and then 5 μL 0.05 N EDTA was added to stop the reaction. Instant thin layer chromatography (ITLC) developed with 0.15 M NH_4OAc was performed to determine the fraction of ^{213}Bi bound to the antibody. The antibody was centrifuged through a Centricon filter to remove unbound ^{213}Bi and HI.

2.4. Exposure of *C. neoformans* to α -particles by ^{213}Bi radiolabeled antibodies

Microcentrifuge tubes (2 mL) were coated with 1% BSA for 2 hours at 4°C. *Cryptococcus* cells were washed twice with PBS at 4,000 RPM for 5 minutes, and then adjusted to 3×10^7 cells/mL. Non-radiolabeled (“cold”) 18B7 antibody was used as control in these experiments. The tubes were incubated at 37°C for 1 hour while shaking. After an hour, samples were centrifuged at 6,000 RPM for 5 minutes, the supernatants were removed, and supernatants and pellets were counted in a γ -ray counter, a process that took about 30 minutes. Then the cells were resuspended in 0.5 mL of PBS and incubated for 3 hours at 37°C followed by overnight incubation at 4°C before being plated for CFUs as described below.

2.5. Clonogenic cell survival assay

Clonogenic survival of *C. neoformans* was determined by plating for CFUs on SAB agar. After each radiation exposure, the cells were diluted to produce an expected number of ~300 CFUs per plate. After two days at room temperature, colonies were visible and were manually counted.

2.6. Estimation of doses to the cell nucleus from ^{213}Bi radiolabeled antibodies

Radionuclide dosimetry for radioimmunotherapy (RIT) is a rapidly evolving field, in which complex and computationally intensive techniques (e.g. Monte Carlo methods) have become common [25-30]. Here our goal was to evaluate: (1) whether the observed killing of *C. neoformans* by ^{213}Bi radiolabeled antibodies would be consistent with predictions based on the α -particle dose to the cell nucleus (or to the cell body) and the linear-quadratic parameters for external-beam α -particles; or (2) whether radiolabeled antibody-mediated cytotoxicity involves important mechanisms which are not activated by external-beam irradiation. To investigate this question, a highly accurate estimate of dose to the *C. neoformans* cell nucleus from radiolabeled antibodies was not required: only a convenient and easily calculated first approximation was needed. For this reason, we chose the analytic dose estimation method described below.

Most of the α -particle dose to the *C. neoformans* cell nucleus delivered by ^{213}Bi radiolabeled antibodies comes from decays of ^{213}Po , a daughter isotope in the ^{213}Bi decay series (Fig. 2). This α -particle dose can be split into two components: (1) dose from decay of radioactive atoms bound to antibodies on the target cell surface (“self-irradiation”), and (2) dose from the radioactive decay on antibodies bound to other cells or suspended in medium (“cross-fire”).

For the first (self-irradiation) scenario, we estimated the dose by integrating over a sphere, which is a reasonable approximation for the shape of the *C. neoformans* cell and cell nucleus [31, 32]. For convenience, we performed the integration in cylindrical coordinates: z-axis (z), radius (r), and angle (ϕ). For our purposes here, where we assumed spherical symmetry, nothing depends on ϕ , so ϕ always = 2π . To set up the integration, we put a source (radioactive atom) emitting a single α -particle (in a random direction) at point $r = 0$, $z = z_s$. For a point inside the sphere with coordinates (r, z), the distance to the source is: $l = (r^2 + (z - z_s)^2)^{1/2}$. The expected fraction of α -particles going through a small disc with area $2\pi r dr$ is the fraction of the solid angle at distance l. This is $\cos(\omega)/(4\pi l^2)$, where the angle ω is between the direction of l and the normal to the disc. It can be found, as ω is an angle with hypotenuse l and side $z_s - z$, that $\cos(\omega) = (z_s - z)/l$. The length of the path along l when we change z by dz is not dz, since the path is not along z but at an angle. So, $dl = dz/\cos(\omega)$, where ω is the same angle as above. Therefore, ω cancels out.

Using these manipulations, we wrote the following integral for the mean energy (E_{surf}) deposited in the cell nucleus per α -decay on a surface-bound antibody, where R_1 is the target radius (e.g. radius of the cell nucleus or of the cell body) and R_2 is the source radius (e.g. radius at which most cell-bound antibodies are concentrated):

$$E_{\text{surf}} = \pi \text{ LET} / (4\pi) \int_{-R_1}^{R_1} \int_0^{R_1^2 - z^2} 1 / (v^2 + (z - R_2)^2) dv dz \quad (1)$$

The solution to Eq. 1 is:

$$E_{\text{surf}} = 1/4 \text{ LET} \left(2 R_1 + \left(R_2^2 - R_1^2 \right) / R_2 \ln [(R_2 - R_1) / (R_2 + R_1)] \right) \quad (2)$$

The mean dose (D_{surf}) to the nucleus from N α -particle decays from surface-bound antibodies is then $N E_{\text{surf}} / (4/3 \rho \pi R_1^3)$, where ρ is the density of the cells and surrounding medium. By substitution, the solution is:

$$D_{\text{surf}} = \frac{3}{16} N \text{ LET} \left(2 R_1 + \left(R_2^2 - R_1^2 \right) / R_2 \ln [(R_2 - R_1) + (R_2 + R_1)] \right) / (\rho \pi R_1^3) \quad (3)$$

To obtain numerical solutions for Eq. 3, we made the following substitutions: $R_1 = R_n$, where R_n is the radius of the *C. neoformans* cell nucleus, and $R_2 = R_a$, where R_a is the radius where most radiolabeled antibodies bind. Alternatively, to estimate the dose to the cell body rather than to the nucleus, we substituted $R_1 = R_c$, where R_c is the radius of the *C. neoformans* cell body. The values for R_n , R_a and R_c are listed in Table 1.

For the second (cross-fire) scenario, we estimated the contribution from α -particles emitted by atoms attached to antibodies bound to other cells or floating in the medium. We used the same basic approach as for calculating D_{surf} , but now we integrated over all extracellular radiation sources in z_s over a spherical shell between radius R_{min} and R_{max} . A schematic representation of the geometry is presented in Fig. 3.

Because the α -particle LET changes as the particle moves along its track, we performed the integration piece-wise in two steps. In the first step, we considered the initial portion of the α -particle track (before the Bragg peak). Here R_{min} is the minimal radius dictated by cellular capsule hindrance, i.e. $\frac{1}{2}$ of the minimum distance between two cell centers. Because *C. neoformans* has a large polysaccharide capsule, antibodies can penetrate deep into the capsule (down to radius R_a), but the outer capsule (radius R_h) somewhat larger than R_a . R_{max} is defined as $R_h + L_i$, where L_i is the length of the initial part of the α -particle track. The values for R_h and L_i are listed in Table 1.

In the second step, we considered the final portion of the α -particle track (which includes the Bragg peak). Here $R_{\text{min}} = R_h + L_i$, and $R_{\text{max}} = R_h + L_i + L_f$, where L_f is the length of the final part of the α -particle track (Table 1). For simplicity, average LET values were used for each of the two parts of the ^{213}Po α -particle track, called LET_i and LET_f , respectively (Table 1). At this stage of the calculation, where only physical dose was estimated, differences in RBE between the initial and final portions of the α -particle track were not considered (but they were considered at subsequent stages, as described below).

Proceeding with dose estimation, we assumed that the number of α -particles in a sub-volume of the spherical shell around the target cell is $dn = 4 N_s \pi z_s^2 dz_s$, where N_s is the concentration of α -particle decays outside the cell. We then wrote the following integral for the mean energy (E_{sol}) deposited in the nucleus from cross-fire α -decays:

$$E_{\text{sol}} = 4 N_s \pi R_2^2 \int_{R_{\text{min}}}^{R_{\text{max}}} E_{\text{surf}} dR_2 \quad (4)$$

The solution to Eq. 4 is:

$$E_{\text{sol}} = \frac{1}{4} \pi N_s \text{LET} \left(W_1 - W_2 + 2 R_1^3 (R_{\text{max}} - R_{\text{min}}) + 2 R_1 (R_{\text{max}}^3 - R_{\text{min}}^3) \right),$$

where : $W_1 = \frac{(R_1 - R_{\text{max}})^2}{(R_1 + R_{\text{max}})^2} \ln \left[\frac{(R_{\text{max}} - R_1)}{(R_{\text{max}} + R_1)} \right],$ (5)

$$W_2 = \frac{(R_1 - R_{\text{min}})^2}{(R_1 + R_{\text{min}})^2} \ln \left[\frac{(R_{\text{min}} - R_1)}{(R_{\text{min}} + R_1)} \right]$$

The mean dose (D_{sol}) to the nucleus from α -particle decays from antibodies not bound to the target cell is then $E_{\text{sol}}/(4/3 \rho \pi R_1^3)$. By substitution, the solution is:

$$D_{\text{sol}} = \frac{3}{16} N_s \text{LET} \left(W_1 - W_2 + 2 R_1^2 (R_{\text{max}} - R_{\text{min}}) + 2 R_1 (R_{\text{max}}^3 - R_{\text{min}}^3) \right) / (\rho R_1^3) \quad (6)$$

To check the accuracy of our integrals (Eqs. 3 and 6), we compared their predictions with published estimates for ^{213}Po doses/decay delivered to spherical cell targets [34], and found that the differences between the methods were small: between 0.1 and 3.8%, thus validating our analytic approach.

The concentrations of bound and unbound antibodies, and their α -particle activities, changed over time throughout radiolabeled antibody exposure due to antibody binding kinetics and isotope decay. Consequently, cumulative doses from radiolabeled antibodies could be found only by integrating time-dependent dose rates (called $dD_{\text{surf}}(t)/dt$ and $dD_{\text{sol}}(t)/dt$ for the self-irradiation and cross-fire dose components, respectively). The first step in estimating the dose rates was estimation of antibody binding kinetics. We used the following differential equations, where t = time, F = concentration of free (unbound) antibodies, B = concentration of antibodies bound to cells, C = cell concentration, K_a = antibody association (binding) constant, and K_d = antibody dissociation constant:

$$dF/dt = -K_a F C + K_d B, \quad dB/dt = K_a F C - K_d B \quad (7)$$

The solutions to Eq. 7 are below, where F_0 is the initial concentration of antibodies at time zero (assumed to be no bound antibodies at that time):

$$B = \frac{-K_a F_0 C (\exp[-(C K_a + K_d)t] - 1)}{(C K_a + K_d)},$$

$$F = F_0 \frac{C K_a \exp[-(C K_a + K_d)t] + K_d}{(C K_a + K_d)} \quad (8)$$

As time approaches infinity, the concentration of bound antibodies (B) approaches the following equilibrium value B_{eq} :

$$B_{\text{eq}} = F_0 K_a C / (K_a C + K_d) \quad (9)$$

These equations (Eqs. 7-9) adequately described our experimental measurements of antibody binding, where the equilibrium fraction of antibodies bound to cells was ~60%, and this equilibrium was reached after ~30 minutes of incubation. Consequently, Eqs 7-9 were used to estimate the values of K_a and K_d (shown in Table 1). The α -particle activities for

the bound and unbound antibodies were estimated by multiplying the respective antibody concentrations by the factor A_f , which represents physical isotope decay:

$$A_f = \ln[2]/T_{1/2} \exp \left[-\ln[2]/T_{1/2} (t+t_0) \right] \quad (10)$$

Here $T_{1/2}$ is the isotope half-life (assumed to be the ^{213}Bi half-life in this case, because the daughter ^{213}Po half-life is orders of magnitude shorter, Fig. 2), and t_0 is the time elapsed between antibody radio-labeling and the beginning of the current experimental phase.

As described above, the radiolabeled antibody experiment consisted of three distinct temporal stages: (1) initial incubation (called “incubation” for simplicity), (2) centrifugation and pelleting (called “pellet”), and (3) final incubation (called “post-pellet”). The cell concentration, the sample volume, and other conditions were assumed to be constant within each of the three stages, but differed considerably between stages (Table 1). Consequently, dose estimation from radiolabeled antibodies was performed separately for each stage. This involved using stage-specific experimental parameters (e.g. cell concentration) and stage-specific initial conditions for antibody binding kinetics (Eq. 7). The dose estimates for each stage were summed.

For the first stage (incubation), we calculated the dose rate for self-irradiation ($dD_{\text{surf}}(t)/dt$) by replacing the number of α -decays per cell (N) in the equation for D_{surf} (Eq. 3) with the per-cell time-dependent α -activity from cell-bound antibodies (i.e. $B A_f/C$, using Eqs. 8 and 10). Then we integrated over time (until the end of stage 1) to get the total self-irradiation dose during the incubation stage. For the cross-fire dose contribution, we used the same basic approach: we replaced the concentration of α -particle decays outside the target cell (N_s) in the equation for D_{sol} (Eq. 6) with the time-dependent α -activity from antibodies bound to other cells or unbound in the medium (i.e. $(B + F) A_f$, using Eqs. 8 and 10) to obtain $dD_{\text{sol}}(t)/dt$, and then integrated over time (until the end of stage 1) to get the total cross-fire dose during the incubation stage.

The same methods were used for the remaining experimental stages. For the second stage (pellet), we assumed the following initial conditions: (1) all unbound antibodies were removed (with the supernatant); (2) only the cell-bound antibodies (which reached equilibrium (B_{eq}) during the previous incubation stage) contributed to radiation dose; (3) the cell concentration (C) increased and became so large that essentially all antibodies remained bound to cells. For the third stage (post-pellet), we assumed the following initial conditions: (1) only the cell-bound antibodies were present at the beginning; (2) the cell concentration (C) decreased again, so antibodies could again freely bind or unbind (according to Eq. 7). For each stage (pellet and post-pellet), the self-irradiation and cross-fire dose contributions were calculated by making the same substitutions and integrations as described above for the first stage (incubation), but using stage-specific initial conditions and parameters.

The dose contribution from β -particles was estimated by solving the differential equations for the concentrations of all radioactive isotopes from the ^{213}Bi decay chain, i.e. ^{213}Bi , ^{213}Po , ^{209}Tl , ^{209}Pb . These equations, which involve simple exponential decay

with well-known half-lives and branching ratios (Fig. 2), are not shown here for simplicity. We assumed that a ^{213}Bi atom attached to a cell-bound antibody remained bound to the cell if it decayed by β -emission. Consequently, its daughter ^{213}Po atom also remained bound to the cell. However, if a ^{213}Bi atom attached to a cell-bound antibody decayed by α -emission, we assumed that it disassociated from the antibody and diffused into the medium. All ^{209}Tl and ^{209}Pb atoms were assumed to be randomly distributed in the medium as well. Consequently, during the first stage (incubation), the entire ^{213}Bi decay chain evolved towards equilibrium, but at the beginning of the second (pellet) stage only the cell-bound ^{213}Bi and ^{213}Po atoms remained (and all other isotopes were removed with the supernatant). During the pellet and post-pellet stages (which together lasted for several ^{213}Bi half-lives), evolution towards equilibrium resumed.

Using the stage-specific and time-dependent concentrations of the three isotopes which decayed by β -emission (i.e. ^{213}Bi , ^{209}Tl , ^{209}Pb), we calculated the β -particle dose by assuming that the energy released by β -decays was uniformly distributed throughout the cells and medium. We neglected edge effects which could lead to some energy loss from the sample, because the total β -particle dose was very small ($<1.5\%$) relative to the α -particle dose and therefore was unlikely to contribute much to ^{213}Bi radiolabeled antibody effects. The even smaller contribution from γ -emissions was neglected.

2.7. Estimation of linear-quadratic (LQ) dose response parameters for γ -rays and α -particles

The linear-quadratic (LQ) model is the mechanistically most plausible and commonly used radiobiological model for clonogenic cell survival. Most mechanistic formalisms of DNA repair kinetics reduce to the LQ form as an approximation [35, 36]. At each (i -th) radiation dose (including the control situation with sham-irradiation), the average expected number of CFUs plated was $N(i)$, and the number of counted colonies was $K(i)$. The plating efficiency ($\text{PE}(i) = K(i)/N(i)$) was assumed to have Binomial errors: this is a more realistic assumption than Gaussian errors, particularly when $\text{PE}(i)$ is close to zero or to 1. Then the log likelihood ($\text{LL}(i)$) for a model-predicted plating efficiency ($\text{PE}_{\text{pred}}(i)$) was calculated as follows (omitting data-specific constants):

$$\text{LL}(i) = K(i) \ln[\text{PE}_{\text{pred}}(i)] + (N(i) - K(i)) \ln[1 - \text{PE}_{\text{pred}}(i)] \quad (11)$$

The model was fitted to the data by maximizing the sum of $\text{LL}(i)$ over all values of i (i.e. over all doses).

We used the classical linear-quadratic (LQ) formalism to describe the *C. neoformans* dose responses to tested radiation types. According to this formalism, the predicted plating efficiency is given by the following equation, where $d(i)$ is radiation dose, PE_{bac} is a background plating efficiency parameter, and α and β are LQ parameters:

$$\text{PE}_{\text{pred}}(i) = \exp \left[- \left(\text{PE}_{\text{bac}} + \alpha d(i) + \beta d(i)^2 \right) \right] \quad (12)$$

The best-fit value for parameter PE_{bac} was extracted analytically from the data at zero dose: $PE_{bac} = -\ln[K(1)/N(1)]$, where $K(1)$ is the number of colonies counted and $N(1)$ is the expected number of CFUs plated at zero dose. The best-fit values of α and β were obtained by maximizing the sum of $LL(i)$ over all values of i (i.e. over all doses), as mentioned above. The same approach (using Eqs. 11 and 12) was used on external-beam γ -ray and α -particle data. Because both γ -ray and α -particle experiments were performed in duplicate, distinct values of PE_{bac} were used for the duplicate experiments, while keeping the radiation-related LQ dose response parameters constant across all experiments which used the same radiation type. In accordance with the theory of dual radiation action, the linear dose response parameter (α) was allowed to depend on radiation type (i.e. different values were allowed for γ -rays and α -particles), whereas a common quadratic parameter (β) was assumed for all radiation types [37-39].

The behavior of the log likelihood function near the best-fit parameter values for γ -rays and α -particles is shown graphically in Fig. 4. Confidence intervals (95% CIs) for α and β were estimated by profile likelihood, using the critical contour of the likelihood function. These CIs are not based on a Gaussian approximation, but on the asymptotic χ^2 behavior of the log likelihood distribution.

2.8. Comparison of dose responses for α -particles delivered by external-beam and by ^{213}Bi radiolabeled antibodies

During exposure to radiolabeled antibodies, non-radiolabeled antibodies contribute to some extent to the cytotoxic effects on *C. neoformans* [15, 17]. Consequently, we modeled cell survival after radiolabeled antibody exposure by the following equation, where PE_{bac} is a background plating efficiency parameter, $Ac(i)$ is antibody concentration, q is a parameter for non-radiological cytotoxic effect of antibodies, α and β are the best-fit LQ dose response parameters estimated for external-beam α -particles, and f_d is the conversion coefficient from radiolabeled antibody concentration to “biologically-effective α -particle dose” (which takes into account increased RBE of the α -particle Bragg peak compared with the initial part of the particle track):

$$PE_{pred}(i) = \exp \left[- \left(PE_{bac} + q \cdot Ac(i) + \alpha \cdot Ac(i) \cdot f_d + \beta \cdot (Ac(i) \cdot f_d)^2 \right) \right] \quad (13)$$

In this equation, the unknown parameters, i.e. those which needed to be estimated using radiolabeled antibody data, were PE_{bac} , q and f_d (because α and β have already been estimated from external-beam α -particle data). As described above for external-beam exposure, the best-fit value for PE_{bac} was extracted analytically from the data at zero dose. The best-fit value of q was determined by maximizing the log likelihood for the experiment where several concentrations of non-radiolabeled antibodies were used.

The best-fit value of the only remaining parameter, f_d , was determined by maximizing the log likelihood for the experiment where ^{213}Bi -labeled antibodies were used. Then f_d was used to estimate the ^{213}Po α -particle Bragg peak RBE (relative to the initial part of the particle track) by using the following relationship, where Ac_H is the highest radiolabeled antibody concentration used in the radiolabeled antibodies experiment (Table 1), D_{Tot} is the

physical α -particle dose to the nucleus estimated for this antibody concentration, D_{Bragg} is the physical α -particle dose to the nucleus contributed by the final portion of the α -particle track which contains the Bragg peak, and $\text{RBE}_{\text{Bragg}}$ is the Bragg peak RBE:

$$\left(D_{\text{Tot}} - D_{\text{Bragg}} \right) + D_{\text{Bragg}} \text{RBE}_{\text{Bragg}} = f_d \text{AcH},$$

rearranging to: $\text{RBE}_{\text{Bragg}} = \left(f_d \text{AcH} + D_{\text{Bragg}} - D_{\text{Tot}} \right) / D_{\text{Bragg}}$ (14)

The best-fit predictions of Eq. 13 were compared with radiolabeled antibody data to assess whether or not the values of α and β derived from external-beam α -particle exposures are consistent with radiolabeled antibody effectiveness. Eq. 14 was used to assess whether or not the estimate of $\text{RBE}_{\text{Bragg}}$ is consistent with radiobiological theory and data from mammalian cells.

3. Results

3.1. *C. neoformans* cell survival after γ -ray or α -particle irradiation

The standard LQ formalism provided reasonable fits to *C. neoformans* clonogenic cell survival curves for both γ -rays and external-beam α -particles (Fig. 5). In contrast, a purely linear dose response model (with separate α values for α - and γ -radiations, but with the β coefficient set to zero), was statistically inferior: its fit to the data was worse by 155 Akaike information criterion (AIC) units, suggesting that the linear model has essentially zero support from the data compared with the LQ model [40, 41]. The best-fit LQ parameter values (Table 3) are approximately two orders of magnitude smaller than those typical for mammalian cells [42-44], indicating the radioresistance of *C. neoformans* to both γ -rays and α -particles.

Using the proportionality between dose and α -particle fluence, i.e. $d = f \text{LET} / \rho$, where d is dose (Gy), f is fluence (particles/ μm^2), LET is linear energy transfer (J/ μm), and ρ is the cell density (kg/ μm^3), and substituting parameters listed in Table 1, we estimated that there were on average 0.12 α -particle traversals through the *C. neoformans* cell nucleus per Gy of external beam exposure. Consequently, the survival curve parameters for α -particles were converted to units of traversals through the nucleus: the linear term $\alpha = 0.089 \text{ traversals}^{-1}$, the quadratic term $\beta = 1.0 \times 10^{-3} \text{ traversals}^{-2}$. These numbers translated into predictions that 91% of *C. neoformans* cells survive 1 traversal through the nucleus, and 11% survive 20 traversals.

For mammalian cell lines, the α coefficient for α -particle traversals through the cell nucleus is typically 3-5 times larger than for *C. neoformans*, and the β coefficient is usually not significantly different from zero [45-47]. This suggests that *C. neoformans* is more radioresistant than mammalian cells even when differences in size of the nucleus are taken into consideration. The radioresistance of *C. neoformans* makes the contribution to cell death from interactions between multiple α -particle traversals per nucleus apparent – this contribution is represented by the β LQ parameter. In contrast, this contribution is “masked” in mammalian cells, which are usually killed by smaller numbers of traversals.

The relative biological effectiveness (RBE) for α -particles vs γ -rays, estimated for low radiation doses using the ratio of linear dose response components (α values), was 4.47 for *C. neoformans* (Table 3). At an α -particle dose of 59.8 Gy, which was predicted to produce 50% cell survival, the RBE decreased to 2.53. At an α -particle dose of 304.9 Gy, which was predicted to produce 1% cell survival, the RBE decreased further to 1.60. These RBE values are within the range reported for mammalian cells at comparable levels of cell survival [48-50]. These results suggest that in *C. neoformans*, as in mammalian cell lines, repair of α -particle induced damage, e.g. complex DNA double strand breaks (DSBs), is more difficult than repair of γ -ray induced damage (e.g. simpler DSBs).

3.2. Estimated radiation doses from ^{213}Bi radiolabeled antibodies

The estimated α -particle doses to the *C. neoformans* cell nucleus, or to the cell body, from the highest tested concentration of ^{213}Bi -labeled capsule-specific antibody are shown in Table 2. The self-irradiation dose contribution (i.e. the dose from α -decays on antibodies bound to target cell surface) was highest during the first (incubation) stage of interaction with radiolabeled antibodies, before physical isotope decay reduced antibody α -activity substantially. The dose to the cell body was somewhat larger than the dose to the nucleus because the radioactive atoms were distributed within the polysaccharide capsule, close to the cell surface.

However, self-irradiation did not account for the majority of the α -particle dose. When cells were brought close together by centrifugation and removal of supernatant during the pellet stage of radiolabeled antibody experiments, cross-fire effects became very important. Both the initial part of the α -particle track (before the Bragg peak), and the final part (which includes the Bragg peak region) contributed substantially (Table 2).

The total physical α -particle dose to the *C. neoformans* nucleus at the highest tested antibody concentration was 66.5 Gy (Table 2). The Bragg peak region of the ^{213}Po α -particle track accounted for about 51% of this dose (33.8 Gy). The best-fit value for the Bragg peak RBE, relative to the initial portion of the α -particle track, was 1.71 (95% CI: 1.46, 1.98). Relative to γ -rays, the Bragg peak RBE was 7.66 (95% CI: 6.32, 9.04) (Table 3). This RBE value is reasonable, considering that maximum LET in the Bragg peak was >3-fold higher, than in the initial part of the ^{213}Po α -particle track [26].

When the Bragg peak RBE was taken into consideration, the “biologically-effective α -particle dose” from radiolabeled antibodies became $(66.5 - 33.8) + 33.8 \times 1.71 = 90.6$ Gy. This high value does emphasize the inherent effectiveness of α -emitter radiolabeled antibodies, and their ability to kill even highly radioresistant pathogens.

3.3. *C. neoformans* cell survival after treatment with ^{213}Bi radiolabeled antibodies

When the increased RBE of the α -particle Bragg peak was taken into consideration, observed cell survival of radiolabeled antibody-treated *C. neoformans* was consistent with predictions based on the α -particle dose to the cell nucleus and the linear-quadratic parameters estimated for external-beam α -particles (Fig. 6). However, non-radiological cytotoxic effects of antibodies (represented by parameter q in Eq. 13) were also present. The

best-fit value of q was 3.70 (95% CI: $3.18, 4.26$) $\times 10^{-3}$ mL/ μ g, which indicated that non-radiological antibody effects accounted for up to 23% of *C. neoformans* cell death.

Thus, our modeling formalism, which assumed that the dose responses for α -particles delivered by external beam, and by ^{213}Bi radiolabeled antibodies, could be described by the same set of linear-quadratic parameters, and that radiological and non-radiological antibody effects were strictly additive, demonstrated reasonable agreement with the data. This result, which suggests that the radiobiological effectiveness of α -particles on *C. neoformans* depends mainly on the dose to vulnerable cell structures, rather than on delivery mode (e.g. external-beam or radiolabeled antibodies), is generally consistent with data on mammalian cells [42, 43, 49, 51].

4. Discussion

To the best of our knowledge, this study provides the first quantitative investigation of how a radioresistant eukaryote *Cryptococcus neoformans* responds to densely-ionizing radiation (α -particles) delivered by two different modes (by external beam or by a radiolabeled monoclonal antibodies), and compares these responses with those induced by sparsely-ionizing radiation (γ -rays). The α -particle LET range explored by our study is quite broad: approximately 64 – 220 keV/ μ m (depending on particle energy and distance traveled along the track). The organism chosen for the study, *Cryptococcus neoformans*, is an important human pathogen [18, 19], and is therefore a valuable model system.

In the past we and others performed experiments showing the susceptibility of *C. neoformans* to α -particle RIT and its resistance to external γ -rays, and here we compared the α -particle RIT with the most relevant (in this case) type of external radiation - an α -particle beam. In addition, we performed dosimetry calculations which, for the first time, estimated: (1) the magnitude of α -particle doses to the nucleus and/or cell body which are needed to kill *C. neoformans* by RIT and (2) the RBE of α -particles (in relation to γ -rays) for a human fungal pathogen. These results help to explain the *in vivo* efficacy of α -particle RIT in experimental fungal infections, which prolongs the survival of the infected animal: the radiolabeled antibodies, which bind very specifically to fungal cells, produce localized α -particle doses to the fungal cells which are sufficiently high to kill these cells, while more distant host cells remain essentially unexposed and unharmed [15-17, 52]. In contrast, external-beam irradiation would target both fungal and host cells indiscriminately, and would have no therapeutic value for fungal infections. This phenomenon is similar to observation from cancer RIT which delivers lower doses to the tumor than external-beam radiation therapy, but is nevertheless effective in slowing down or abrogating tumor growth because of more specific radiation damage to tumor cells [53].

The limitations of the study include relatively narrow cell survival range ($>10\%$ survival) after radiation exposure, and simplified dose estimation and dose response modeling methods. For external-beam irradiation, the limited cell survival range resulted from the requirement to keep radiation exposure times short enough, given the dose rates available. For radiolabeled antibody exposure, it resulted from the requirement to maintain antibody concentration low enough to ensure specific antibody binding. However, the data collected

within this survival range were sufficiently detailed to provide information for meaningful modeling and statistical analysis of the radiation dose response. In other words, the data were sufficient for the goal of the paper - to provide an initial assessment and comparison of the cytotoxicities of α - and γ -radiation on *C. neoformans*. Because a *C. neoformans* cell is very close to a sphere in shape [31, 54], the assumptions of spherical symmetry for the dose estimation calculations are biologically reasonable for this organism. Consequently, we believe that the study limitations identified above do not affect the general conclusions of the study.

Our results show that *C. neoformans* is very resistant not only to sparsely-ionizing, but also to densely-ionizing radiation: depending on growth conditions and culture stage, it can survive 100- to 1000-fold higher radiation doses than mammalian cells [15]. Even though only 1 to 10% of the capsule-specific antibody molecules are actually radiolabeled with ^{213}Bi , treatment with such antibodies overcomes the radioresistance of *C. neoformans* primarily by producing highly localized α -particle doses (tens of Gy) to target cell structures, including the nucleus. Such doses are sufficient to kill even radioresistant targets like *C. neoformans*, while resulting in very low "collateral damage" to the surrounding mammalian cells [15-17, 52]. These doses provide a plausible explanation for why radiolabeled antibodies were shown to be more effective against *C. neoformans* than pharmacological anti-fungal treatment [17].

In our examination of the effects of radiolabeled antibodies on *C. neoformans* cell survival, we cannot rule out a synergistic effect of antibodies and radiation, but a purely additive effect (which represents the simplest assumption, and was therefore used here) was sufficient to describe the data and produced plausible parameter estimates. For *in vivo* applications of radiolabeled antibody therapy against fungal pathogens, which were successfully tested in mice [15, 17], much lower amounts of the antibodies (30-50 μg per mouse) were used in comparison with the "cold" antibody therapy which uses milligram amounts. In addition, *in vivo* 30-50 μg of antibody never produced any effect on the mouse survival or fungal burden, and the effect of the cold antibody only manifests itself *in vitro* [15, 17].

Thus, we demonstrate that the dosimetry results can explain why exposure of fungal cells to the radiolabeled antibody produces very high localized doses to the fungal cells, and can successfully fight fungal infection both *in vitro* and *in vivo* [15-17] even though fungal pathogens are quite radioresistant. More detailed biological investigation of the mechanisms of *C. neoformans* radioresistance and radiation responses is beyond the scope of the current paper, but it is the priority for our future research.

The α -particle RBE, relative to γ -irradiation, estimated from our study is within the range reported for mammalian cells [48-50]. This is consistent with current knowledge of DNA DSB repair mechanisms in *C. neoformans*: unlike the well-researched yeast *Saccharomyces cerevisiae*, which often uses homologous recombination (HR) to repair DSBs, *C. neoformans* usually grows in haploid form and uses Ku-mediated non-homologous end joining (NHEJ) as the dominant DSB repair pathway [55, 56]. Mammalian cells, particularly during G0 or G1 phases of the cell cycle, also rely heavily on NHEJ for DSB repair [57, 58].

The similarity in preferred DSB repair mechanisms between *C. neoformans* and mammalian cells may explain why their intron/exon ratios are also similar, whereas introns are rare in *S. cerevisiae* [59]. The combination of these results suggests that the responses of *C. neoformans* to different radiation types conform to general patterns of dose response shape and LET-dependence of RBE observed in mammalian cell radiobiology, and that the efficiency of DNA repair mechanisms may be reasonably similar in *C. neoformans* and in mammalian cell lines.

5. Conclusion

We quantified and compared the effects of densely-ionizing α -particles (delivered either by external beam or by ^{213}Bi -labeled monoclonal antibodies), and sparsely-ionizing ^{137}Cs γ -rays, on a radioresistant pathogenic fungus *Cryptococcus neoformans*. Fungal cell killing by radiolabeled antibodies was consistent with predictions based on the α -particle dose to the cell nucleus and the linear-quadratic parameters for external-beam α -particles. These results quantify for the first time the degree of a human fungal pathogen resistance to densely-ionizing radiations, and show how this resistance can be overcome with the application of fungus-specific radiolabeled antibodies which produce very high and localized α -particle doses to the fungal cells.

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Fig. 1. Schematic, rotated depiction of external-beam α -particle irradiation of *C. neoformans* cells in Mylar-bottom dishes. Details are described in the main text.

Fig. 2.

Decay series for ^{213}Bi . Half-lives ($T_{1/2}$), branching ratios, and average particle energies were taken from reference [60].

Fig. 3. Schematic representation of the geometry used to calculate α -particle doses to the *C. neoformans* cell body and nucleus delivered by radiolabeled antibodies. The variables r , z and l are discussed in the main text.

Fig. 4.

Behaviors of the log likelihood function in parameter space near the best-fit linear-quadratic parameter values for external-beam α -particles and γ -rays. As discussed in the main text and in Table 3, the linear-quadratic parameter β was held in common for all radiation types. Contours delineate parameter regions with the same log likelihood. The inner contour encloses the region which contains the best-fit parameter values.

Fig. 5. *C. neoformans* clonogenic survival curves for external-beam α -particles and γ -rays. Different symbol types of each color represent separate repeat experiments. Curves represent best-fit linear-quadratic dose responses with parameters discussed in the main text. Error bars are 95% confidence intervals.

Fig. 6. The effects of ^{213}Bi radiolabeled antibodies, and non-radiolabeled antibodies, on clonogenic survival of *C. neoformans*. Curves represent best-fit dose responses. As discussed in the main text, the values of the linear-quadratic radiation-related parameters used to generate the predicted survival curve for ^{213}Bi radiolabeled antibodies are the same as those estimated for external-beam α -particles. Error bars are 95% confidence intervals.

Table 1

Parameters used in the calculations for estimating cellular doses from ^{213}Bi radiolabeled antibodies.

Parameter	Meaning	Value	Reference
Rn	Radius of cell nucleus	0.68 μm	[54]
Rc	Radius of cell body	4.0 μm	This study
Ra	Radius where most radiolabeled antibodies bind	5.5 μm	This study and [33]
Rh	Radius of hindrance between cells	7.0 μm	This study
Ka	Antibody association constant	$1.86 \times 10^5 \mu\text{m}^3/\text{h}$	This study
Kd	Antibody dissociation constant	$1.99 \mu\text{m}^3/\text{h}$	This study
C	Cell concentrations during incubation, pellet, and post-pellet stages of radiolabeled antibody experiment	1.6×10^7 , 6.4×10^8 , 8.0×10^6 mL^{-1}	This study
AcH	Highest radiolabeled antibody concentration	16.42 μg per 0.15 mL tube	This study
T	Durations of the incubation, pellet, and post-pellet stages of radiolabeled antibody experiment	1.0, 0.5, 4.0h	This study
π	Density of cells and surrounding medium	1.04 g/cm^3	This study
LET	LET of external-beam α -particles	72 $\text{keV}/\mu\text{m}$	SRIM software
LET ₀	Initial LET of ^{213}Po α -particle (for cell self-irradiation calculations)	64 $\text{keV}/\mu\text{m}$	SRIM software
LET _i	Average LET of ^{213}Po α -particle over the first 50 μm of track	70.6 $\text{keV}/\mu\text{m}$	[26]
LET _f	Average LET of ^{213}Po α -particle over the last 35 μm of track (Bragg peak region)	143.5 $\text{keV}/\mu\text{m}$	[26]
L _i	Initial part of ^{213}Po α -particle track (before the Bragg peak)	50 μm	[26]
L _f	Final part of ^{213}Po α -particle track (Bragg peak region)	35 μm	[26]

Notes. Range and LET parameters are reported for ^{213}Po α -particles, rather than for ^{213}Bi α -particles, because the former contribute the most to cellular doses delivered by ^{213}Bi radiolabeled antibodies. SRIM software is available from <http://www.srim.org>.

Table 2

Estimated cellular doses to *C. neoformans* from ^{213}Bi radiolabeled antibodies.

Experi- mental stage	α -particle dose (Gy) from decays on antibodies bound to target cell surface		α -particle dose (Gy) from decays on antibodies bound to other cells or suspended in medium				Total α - particle dose (Gy)		β - particle dose (Gy)
			Contribution from initial 50 μm of α - particle track		Contribution from terminal 35 μm of α - particle track (Bragg peak region)				
	Nucleus	Cell body	Nucleus	Cell body	Nucleus	Cell body	Nucleus	Cell body	Whole cell
Incubation	4.83	5.49	3.64	3.67	4.85	4.85	13.31	14.01	0.48
Pellet	1.60	1.82	21.45	21.62	28.57	28.58	51.62	52.03	0.11
Post-pellet	0.91	1.04	0.28	0.28	0.37	0.37	1.56	1.69	0.33
Total	7.34	8.35	25.37	25.57	33.79	33.80	66.50	67.72	0.92

Notes. As discussed in the main text, range and LET parameters for ^{213}Po α -particles, rather than for ^{213}Bi α -particles, were used to generate the dose estimates because the former contribute the most to cellular doses delivered by ^{213}Bi radiolabeled antibodies. β -particle doses were assumed to be uniform to all cell structures.

Table 3

Linear-quadratic model parameters (α , β) for clonogenic survival of *C. neoformans* exposed to different radiation types and delivery modes.

Radiation type	Radiation source	α ($\times 10^{-2}$ Gy $^{-1}$)		β ($\times 10^{-5}$ Gy $^{-2}$)		RBE	
		value	95% CIs	value	95% CIs	value	95% CIs
γ -rays	External beam	0.24	0.22 0.26	1.44	1.35 1.53	1.00	1.00 1.00
α -particles	External beam	1.07	1.03 1.12	1.44	1.35 1.53	4.47	4.06 4.89
	^{213}Po , first 50 μm of track	1.07	1.03 1.12	1.44	1.35 1.53	4.47	4.06 4.89
	^{213}Po , last 35 μm of track	1.83	1.55 2.13	1.44	1.35 1.53	7.66	6.32 9.04

Notes: RBE refers to relative biological effectiveness of different radiation types at low doses, relative to γ -rays.