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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: $\alpha = 0.24 \times 10^{-2}$ Gy^{-1} for γ-rays and 1.07×10^{-2} Gy^{-1} for external-beam α-particles, and $\beta = 1.44 \times 10^{-5}$ 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.
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.

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 (^{4}He 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

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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 × 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 ^{4}He 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.

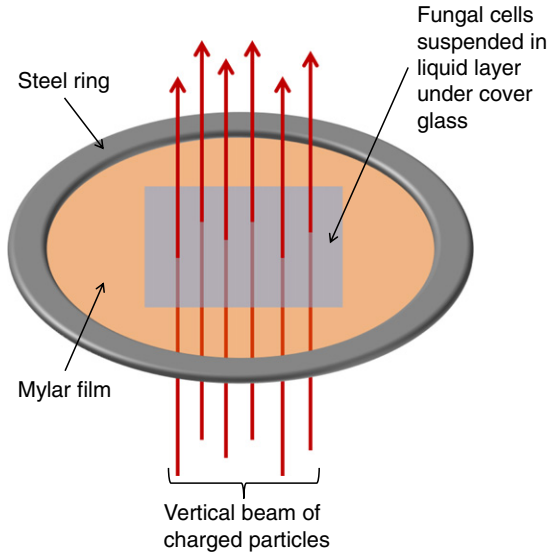

Fig. 1. Schematic, rotated depiction of external-beam α-particle irradiation of *C. neoformans* cells in Mylar-bottom dishes. Details are described in the main text.

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 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.

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 Ref. [33]
Rh	Radius of hindrance between cells	7.0 μm	This study
Ka	Antibody association constant	1.86×10^5 μm^3/h	This study
Kd	Antibody dissociation constant	1.99 μm^3/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, 4.0 h	This study
ρ	Density of cells and surrounding medium	1.04 g/cm^3	This study
LET	LET of external-beam α-particles	72 keV/μm	SRIM software
LET_0	Initial LET of ^{213}Po α-particle (for cell self-irradiation calculations)	64 keV/μm	SRIM software
LET_i	Average LET of ^{213}Po α-particle over the first 50 μm of track	70.6 keV/μm	[26]
LET_f	Average LET of ^{213}Po α-particle over the last 35 μm of track (Bragg peak region)	143.5 keV/μm	[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.