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Organ-Dependent Variation of Capsule Thickness in *Cryptococcus neoformans* during Experimental Murine Infection

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In studies of murine infection, the capsule thickness of *Cryptococcus neoformans* varied depending on the organ. The relative order of thickness was as follows: lung > brain > in vitro isolates. The differences in capsule thickness suggest that there are organ-related differences in the expression of genes responsible for capsule thickness.

Cryptococcus neoformans causes disease in 6 to 8% of AIDS patients (7). The fungus has a polysaccharide capsule composed primarily of glucuronoxylomannan (GXM) (5) which is important for virulence (1, 6). In the 1950s, Littman's studies revealed that the capsule thickness of *C. neoformans* increases during infection (15). However, when organisms recovered from organ tissue are transferred to culture media, capsule thickness usually decreases (15, 16). While studying the fate of cryptococci in vivo, we noted differences in the capsule thicknesses of cryptococci in brain and lung homogenates. To investigate this, we measured capsule thicknesses using combined India ink staining, fluorescent-dye staining, and indirect immunofluorescence by microscopy supported by computer analysis.

(The data presented in this paper are from a thesis to be submitted by J. Rivera in partial fulfillment of the requirements for a Ph.D. from the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y. These data were presented at the 98th General Meeting of the American Society for Microbiology, Atlanta, Ga., 1998.)

C. neoformans 24067 was obtained from the American Type Culture Collection (ATCC, Rockville, Md.) and grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.) for 24 h at 30°C. The cells were washed three times with sterile phosphate-buffered saline (PBS; pH 7.4) and counted with a hemacytometer. The immunoglobulin G1 (IgG1) monoclonal antibody (MAb) 2H1 has been described elsewhere (2, 3). Levels of GXM in brain and lung homogenates from infected mice were measured by double-sandwich enzyme-linked immunosorbent assays (4, 11, 13). Organ GXM contents were normalized for the number of CFU in each organ.

Six- to eight-week-old female A/JCr, SCID/NCr, and BALB/c mice (National Cancer Institute, Frederick, Md.) were infected intratracheally as described elsewhere (9). The infecting inocula for A/JCr, SCID/NCr, and BALB/c mice were 10^7, 10^5, and 10^5 organisms, respectively. Two weeks later, the mice were killed by cervical dislocation, and the brain and lung tissues were homogenized. Aliquots of the homogenates were spread on Sabouraud dextrose agar plates (Difco Laboratories). Individual colonies were grown in Sabouraud dextrose broth for subsequent infections. The sequence of infection with cultures from harvested organs is illustrated in Fig. 1A.

Capsule dimensions in organ homogenates and cultures were measured. Twenty microliters of MAb-stained (18) brain or lung homogenate was placed on a slide with 20 μl of 1% Uvitex 3BSA–PBS (Ciba-Geigy, Greensboro, N.C.). Coverslips were applied, and a small drop of India ink (Difco Laboratories) was added. The slides were viewed with an Olympus IX 70 microscope (Olympus America, Inc., Melville, N.Y.) with ×60 numerical aperture. 1.4 optics equipped with standard fluorescein isothiocyanate and 4′,6-diamidino-2-phenylindole (DAPI) filters. Images were collected with a Photometrics KAF 1400-cooled, charge-coupled device camera (Tucson, Ariz.) on a Power Macintosh 8500 (Apple Computer, Austin, Tex.) with I. P. Lab Spectrum software (Scanalytics, Fairfax, Va.). The thickness of the *C. neoformans* capsule and cell body was measured by tracing the circumference of the whole organism and cell body at the equatorial plane. Calculations of area and volume were partially automated by using macros written within the public-domain software NIH-Image (National Institutes of Health, Bethesda, Md.). All data were analyzed by the Student *t* test and the Kruskal-Wallis test (Primer; McGraw-Hill, Inc., New York, N.Y.). P values of <0.05 were considered significant.

Uvitex 3BSA binds chitin in the yeast cell wall (14) and was used to delineate the cell wall. The outer boundary of the capsule was established by combining India ink staining and indirect immunofluorescence with GXM-specific MAbs (Fig. 1B). To determine if India ink or MAb altered capsule thickness, yeast cells were suspended in India ink with Uvitex 3BSA and in MAb with Uvitex 3BSA. Measurements of capsule thickness were the same in both instances (data not shown). To determine if the tissue homogenate influenced capsule thickness, yeast cells were suspended and cultured in brain and lung homogenates of noninfected mice. Capsule thickness did not change (data not shown).

C. neoformans cells, which were grown in vitro and used for initial infection, had an average capsule thickness of 2.8 ± 1.4 μm. Yeast cells in tissue homogenates had significantly larger capsules, with an average capsule thickness among lung organisms of 20.0 ± 6.1 μm, whereas organisms isolated from the brain had an average capsule thickness of 8.4 ± 3.9 μm (Fig. 2) ($P < 0.001$). Uvitex 3BSA and MAb 2H1 staining revealed differences in the capsule thicknesses of organisms in lung and brain tissues and in vitro (Fig. 3). In addition, organisms isolated from the lungs of SCID/NCr and BALB/c mice had average capsule thicknesses of 18.5 ± 8.3 μm and 17.9 ± 7.6 μm, respectively ($P = 0.648$). The average capsule volumes for organisms in lung and brain tissues and under in vitro condi-

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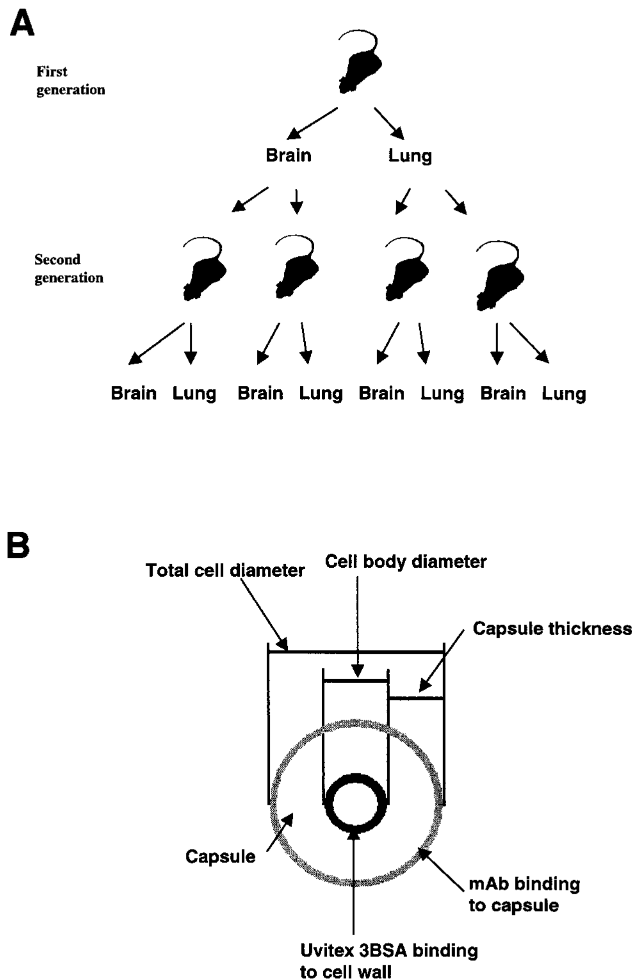

FIG. 1. (A) Illustration of experimental design. One mouse was infected intratracheally with *C. neoformans*. Brain and lung organisms were harvested and stained with India ink, 1% Uvitex 3BSA, and 50 μg of MAb 2H1 per ml. Brain and lung colonies were recovered from Sabouraud dextrose agar plates, grown in Sabouraud dextrose broth, and used to infect subsequent mice as shown. The scheme shown here, involving a total of seven mice (two mice from the first passage and five mice from the second passage), was done twice. (B) Illustration of *C. neoformans* and staining location. Diagram shows Uvitex 3BSA binding to the cell wall and MAb binding to capsule, allowing measurement of total cell and cell body diameter. Volume ($4/3\pi r^3$) and area (πr^2) were determined. Capsule thickness and volume were determined by subtracting the diameter and volume of the cell body from total cell diameter and volume.

tions were $1.1 \times 10^5 \pm 6.6 \times 10^4$ μm^3, $1.7 \times 10^4 \pm 2.7 \times 10^4$ μm^3, and $3.3 \times 10^3 \pm 1.7 \times 10^3$ μm^3, respectively.

To explore whether the differences in capsule thickness reflected inherent characteristics of yeast cells in these tissues or a response to the tissue environment, mice were infected with *C. neoformans* recovered from brain and lung homogenates. Yeast cells from mice infected with isolates recovered from lung homogenates again had capsules in the lung that were significantly larger than those in the brain ($P < 0.001$) (Fig. 2). In addition, yeast cells from mice infected with isolates recovered from brain homogenates had capsules in the lung that were significantly larger than those in the brain ($P < 0.001$) (Fig. 2). Hence, the changes in capsule thickness were reversible. Organisms isolated from the brain and lung had the same average capsule thickness when they were grown in vitro (Fig. 2). Despite the source of the isolate used for infection, the relative order of capsule thickness was as follows: lung > brain > in vitro-grown isolates. The concentrations of polysaccharide in brain and lung tissues were $8.8 \times 10^{-1} \pm 1.0$ μg/CFU and $1.8 \times 10^{-3} \pm 1.6 \times 10^{-3}$ μg/CFU, respectively ($P < 0.001$). The average capsule thicknesses in minimal media with and without 10 μM FeEDTA were 3.2 ± 1.1 μm and 8.6 ± 1.9 μm, respectively ($P < 0.001$). In contrast, no differences in capsule thickness were observed when strain 24067 cells were grown in Dulbecco modified Eagle medium with various concentrations of $NaHCO_3^-$ and 5% CO_2 (data not shown).

Comparison of *C. neoformans* cells from media under in vitro conditions and from organ homogenates revealed large differences in capsule thickness. The capsule thicknesses of cells grown in media were consistently smaller than those of cells recovered from lung and brain homogenates. Capsule thickness changes were reversible in that cells recovered from organ homogenates and grown in media reverted to cells with smaller capsules. Similarly, transfer of cells from media to in vivo conditions by infecting mice reproducibly resulted in cells with larger capsules, confirming the classic study of Littman in the late 1950s (15). Since mice were infected intratracheally, we considered the possibility that the difference in capsule thickness reflected preferential extrapulmonary dissemination of cells with smaller capsules. However, when single colonies were recovered from the brain and reintroduced into mouse lung, organisms with significantly larger capsules were recovered from lung homogenates compared with brain homogenates. Furthermore, single colonies recovered from lung homogenates and reintroduced into mouse lungs disseminated, and the capsules of *C. neoformans* cells in brain homogenates were small. The difference in size was unlikely to reflect an artifactual effect of homogenate products on capsule thickness, because capsule thickness did not change when *C. neoformans* cells were suspended and grown in brain and lung homogenates. The murine immune response did not appear to influence capsule thickness, since organisms from infected SCID/NCr and BALB/c mice had similar capsule thickness differences between brain and lung homogenates. Thus, the differences in capsule thickness are significant and reflect growth conditions in the lung and the brain.

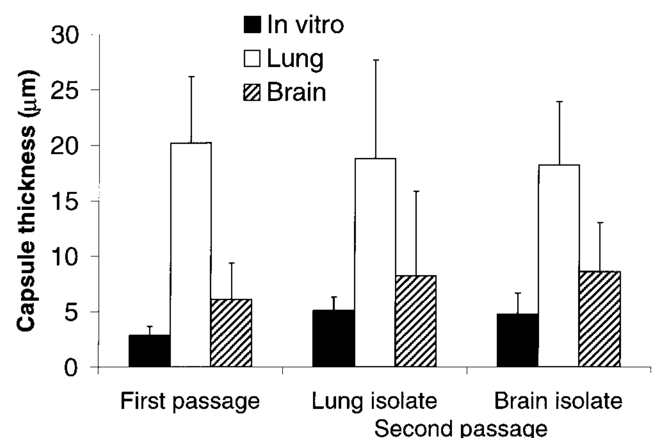

FIG. 2. Capsule thickness of yeast cells isolated from lung and brain tissues of infected mice. Bars denote average capsule thicknesses from organisms isolated from lung and brain tissues. Error bars denote standard deviations. The differences between organisms isolated from lung and brain homogenate were seen in each of the seven mice analyzed. For each measurement, the numbers of organisms analyzed ranged from 6 to 29 from brain homogenate and from 12 to 17 from lung homogenate. $P_1 < 0.001$ for comparison of capsule thicknesses from brain and lung isolates with that of in vitro-grown isolate ATCC 24067; $P_2 < 0.001$ for comparison between capsule thicknesses from brain and lung isolates.

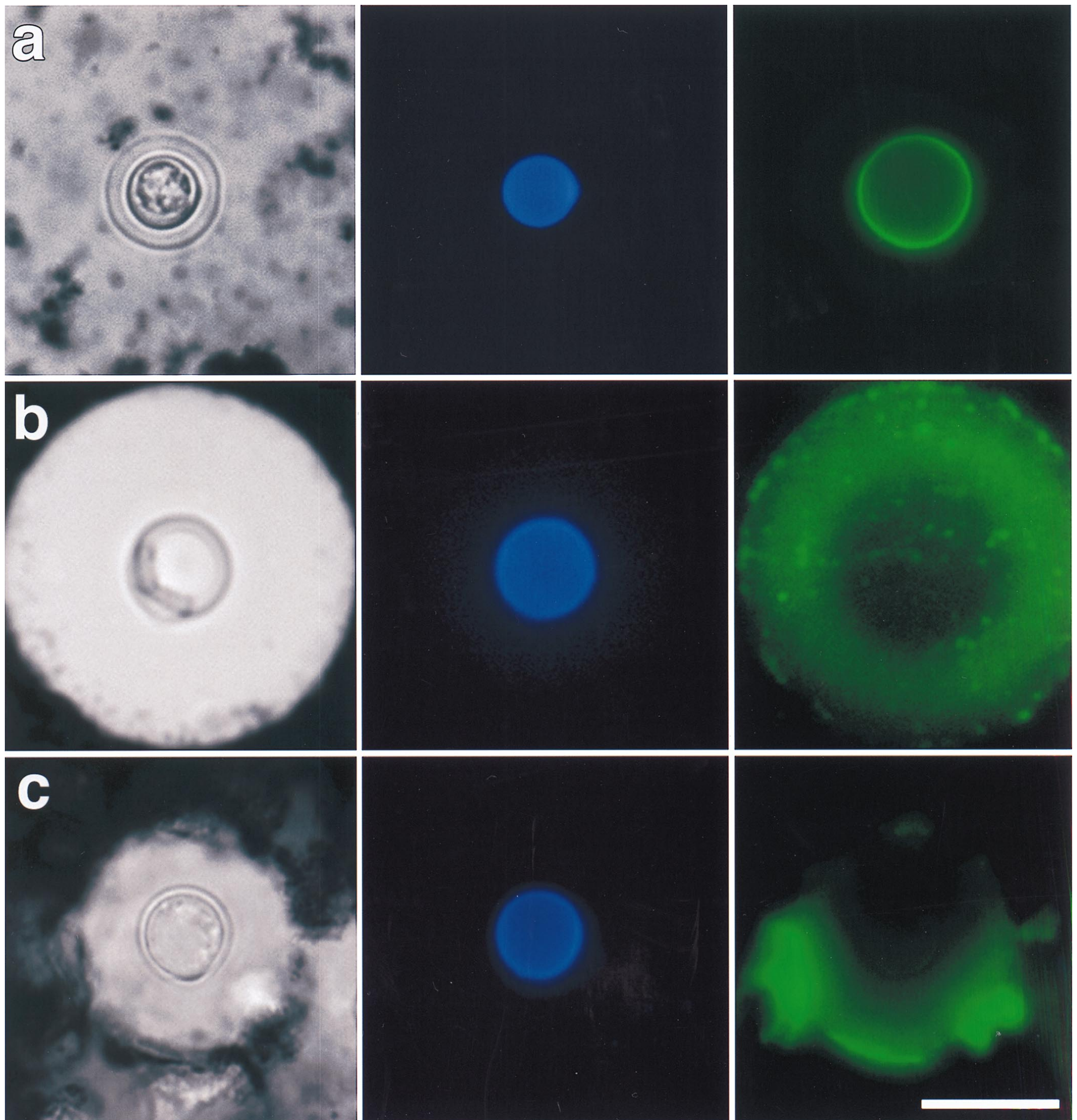

FIG. 3. Indirect immunofluorescence and India ink staining of representative *C. neoformans* cells grown in vitro (a) and isolated from lung (b) and brain (c) tissues of infected mice. Organ suspensions were stained with India ink (left panels), 1% Uvitex 3BSA (center panels), and 50 μg of IgG1 MAb 2H1 per ml (right panels). Scale bar (10 μm) applies to all panels.

The mechanism responsible for the differences in capsule thickness for cells in lung and brain is unknown. Phenotypic variation has been described for *C. neoformans* and is accompanied by changes in multiple cellular characteristics, including capsule thickness and cell size (10). The observation that growth in media with a high iron concentration resulted in cells with smaller capsules may be relevant to this effect, since the concentration of iron in brain tissue is greater than that in lung tissue (8, 17). In contrast, CO_2 is less likely to be a factor, since the capsule thickness of our strain was not affected by HCO_3^-/CO_2 and the concentrations of CO_2 in brain and lung tissues are equivalent (12). Enhanced shedding of polysaccharide in brain tissue is suggested by the observation that the level of soluble GXM in brain tissue was greater than that in lung tissue. However, the possibility remains that the quantitative differences in soluble GXM reflect various abilities of the host

to clear polysaccharide from the brain and lung. At this time, we cannot distinguish whether the observed phenotypic switch resulted from organ-related nutritional differences or enhanced GXM shedding. Furthermore, these findings were obtained with *C. neoformans* ATCC 24067, and future work is needed to establish whether the same phenomenon occurs with other strains. Nevertheless, organ-related differences in capsule thickness imply variation in capsule gene regulation in the two organs.

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