Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection

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The pathogenesis of *Cryptococcus neoformans* infection has been studied extensively with respect to inflammatory and pathological changes, but very little information is available regarding the morphology of yeast cells during the course of infection. Electron microscopy of *Cryptococcus neoformans* in murine pulmonary infection revealed increased cell wall thickness with time, but this difference was only partially accounted for by increases in cell diameter. Cell walls of melanized cells were thicker than those of nonmelanized cells 2 h after infection, and the cell wall of yeast became blacker with time, suggesting that melanization contributes to the increased cell wall thickness. Heterogeneous cell populations emerged, with the appearance of giant forms. While for *C. neoformans* ATCC strain 24067 (serotype D) the full spectrum of cell sizes were observed, for strains H99 (serotype A) and 3501 (serotype D) cells were divisible into two populations, giant and micro forms. In contrast to cellular heterogeneity, the epitope recognized by a protective mAb on the capsular glucuronoxylomannan (GXM) was found at all times of infection. Immunoelectron microscopy using mAbs to GXM demonstrated reactivity with intracellular structures, suggesting that synthesis of capsular polysaccharide occurs, at least in part, in the cytoplasm. In summary, the results indicate that: (i) the infection is dynamic with respect to yeast cell morphology; (ii) giant cell forms arise in tissue during the course of infection; (iii) cell walls blacken and thicken during the course of infection, consistent with melanin synthesis during infection; and (iv) GXM epitopes are found in the capsule, cell wall and cytoplasm, consistent with intracellular polysaccharide synthesis. The results indicate that the population of *C. neoformans* cells in tissue is in a highly dynamic state, implying that the immune system must confront cells with varying characteristics during the course of infection.

Keywords: yeast, lung, ultrastructure

INTRODUCTION

Cryptococcus neoformans is the causative agent of cryptococcosis, a life-threatening fungal infection (reviewed by Mitchell & Perfect, 1995). Cryptococcosis is a relatively frequent complication of late stage HIV infection (Currie & Casadevall, 1994) and is associated with other immunosuppressed states, such as haemato-

logic malignancies, collagen vascular diseases and steroid therapy. *C. neoformans* infection is believed to be acquired by inhalation of infectious particles (Levitz, 1991). In the immunocompetent host, the infection is often asymptomatic and limited to the lung. However, in patients with impaired immunity, extrapulmonary dissemination to the central nervous system can occur and meningoencephalitis is the most common clinical presentation of cryptococcosis. Cryptococcosis is currently an incurable infection in patients with AIDS because existing therapy does not eradicate the infection

Abbreviations: L-dopa, L-3,4-dihydroxyphenylalanine; GXM, glucuronoxylomannan; IEM, immunoelectron microscopy.

in the setting of severe immune suppression (Zuger *et al.*, 1986).

C. neoformans infections in both humans and experimental animals are characteristically chronic. The ability of *C. neoformans* to persist in tissue, even in immunologically intact hosts, is not well understood. There is considerable evidence that the inability of the host to eradicate infection results from interference with host defence mechanisms by the capsular polysaccharide, which can be found in copious amounts in tissue (Casadevall & Perfect, 1998). However, it is also possible that the *C. neoformans* population in host tissue undergoes changes that contribute to the inability of the host immune response to clear the infection. For other pathogens, antigenic variation is an important mechanism for evasion of host defences. Recently, *C. neoformans* has been shown to be able to undergo reversible cellular morphological changes by phenotypic switching *in vitro* and, possibly, *in vivo* (Goldman *et al.*, 1998). For *C. neoformans* cells, several ultrastructural studies have provided highly detailed information on the cellular structure, cell wall and capsule (Al-Doory, 1971; Cassone *et al.*, 1974; Mochizuki *et al.*, 1987; Sakaguchi *et al.*, 1993). From analysis of freeze-etched samples, increases in cell wall thickness, cell body size, capsule size and secretory vacuole activity have been noted in comparisons of yeast *in vivo* and *in vitro* (Sakaguchi *et al.*, 1993; Takeo *et al.*, 1973). However, relatively little information is available on the ultrastructure of *C. neoformans* cells during progressive tissue infection, or the site of synthesis of the capsular polysaccharide.

We recently completed a detailed ultrastructural study of pathology and the host response in murine pulmonary cryptococcal infection (Feldmesser *et al.*, 2000a). That study focused on identification of the location of *C. neoformans* replication in tissue and established that this yeast is a facultative intracellular pathogen in murine pulmonary infection. During the completion of that study, we noted differences with time in the morphology of *C. neoformans* cells in the lungs of infected mice. The availability of tissue sections from various stages of infection allowed us to ask if the morphology of the yeast cell changed during the course of infection. Here, we report on the yeast cell morphological changes associated with chronic infection. We analysed changes during the course of infection in yeast cell size, cell wall thickness and in the appearance of the cell wall by light microscopy. Further, we performed immunoelectron microscopy (IEM) using closely related mAbs that bind glucuronoxylomannan (GXM), the major component of the capsular polysaccharide, which demonstrated differences in intracellular binding patterns within serotype D strains and provided insight into sites of polysaccharide synthesis. The results indicate that the morphology of yeast cells varies during infection, implying the occurrence of dynamic changes that may contribute to the ability of this organism to persist in tissue.

METHODS

C. neoformans. ATCC strain 24067 (serotype D) was used for most experiments because it has been used in previous studies of pulmonary pathology (Feldmesser *et al.*, 1998; Feldmesser & Casadevall, 1997). Limited experiments were done using the acapsular strain Cap 67, its parent strain 3501 (serotype D) (Jacobson & Tingle, 1994) and H99 (serotype A), the type strain for *C. neoformans* var. *grubii* (Franzot *et al.*, 1999). Isolates were maintained at -80°C . Cultures were started by inoculation of Sabouraud dextrose broth (Difco) with a loopful of frozen stock and incubated for 48 h at 30°C with moderate shaking. For one experiment designed to look at the appearance of yeast melanized *in vitro*, *C. neoformans* was grown in a defined minimal medium (15 mM glucose, 10 mM MgSO_4 , 13 mM glycine, $3.0\ \mu\text{M}$ vitamin B_1 , pH 5.5) with or without 1.0 mM L-dopa (Sigma) at 30°C for 13 d. Cultures were washed three times in sterile phosphate-buffered saline, counted using a haemocytometer, and the count was confirmed by plating on Sabouraud dextrose agar. One culture of yeast grown and prepared in this manner was fixed in Trump's fixative (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer) and prepared for EM, as described below.

Murine infection. Specific pathogen-free C57BL/6, BALB/c and A/JCr mice were obtained from the National Cancer Institute (Bethesda, MD, USA); 129/SvEv mice were obtained from Taconic Farms (Germantown, NY, USA). Within each experiment, mice were of the same sex. Six to 10 week old mice were anaesthetized with 65 mg sodium pentobarbital kg^{-1} and inoculated intratracheally with 10^4 or 10^6 organisms in 0.05 ml sterile PBS via a midline neck incision using a bent 26 gauge needle attached to a tuberculin syringe. The incision was then sutured with 5-0 silk (Feldmesser & Casadevall, 1997). The higher inoculum was used for experiments in which mice were killed prior to 24 h after infection to facilitate visualization of yeast cells in tissue by EM. The lower inoculum was used for experiments in which mice were killed 24 h or later after infection, except where stated. In the following sets of experiments, mice of the strain indicated in parentheses were infected concurrently with strain 24067, except where indicated, and were killed at: (i) 5 min or 2 h after infection (BALB/c); (ii) 24 h, 48 h, 7 d or 28 d after infection (C57BL/6); (iii) 13 d after infection with 10^4 or 10^6 yeast cells (129/SvEv); (iv) 14 d after infection (A/JCr); (v) 14 d after infection (C57/BL6); (vi) 24 h after infection with strains 24067, Cap 67 or 3501 (C57/BL6); (vii) 14 d after infection with strain 3501 (C57/BL6); (viii) 2 h or 14 d after infection with strain H99 (C57/BL6); and (ix) 2 h after infection with strain 24067 grown in minimal medium with or without L-dopa (C57/BL6). In each experiment, two mice were studied for each group. Overall, this study includes data from 36 mice. At the times indicated in individual experiments, mice were killed by cervical dislocation. Their lungs were removed and fixed in Trump's fixative for EM.

Microscopy. For EM, tissue blocks and cells were post-fixed with 1% osmium for 1 h, dehydrated in ascending ethanol (30–100%), cleared in two changes of acetonitrile, and then infiltrated with and embedded in araldite-epon, as described by Feldmesser *et al.* (1997). After light microscopic review of $1\ \mu\text{m}$ toluidine-blue-stained sections, ultrathin sections of selected regions were stained with uranyl acetate and lead citrate and examined with a JEOL 100S or 100 CX electron microscope. A minimum of five noncontiguous grids were imaged for each mouse and the data were pooled for analysis. At least 22% of the yeast cell measurements came from each

Table 1. Cell wall thickness of *C. neoformans* strain 24067 *in vitro* and at various times of infection

Condition	Cell wall thickness*				Mean cell wall thickness/cell diameter	
	Mean $\pm$ SD (n)†	Median	Range	P‡	$\pm$ SD (n)†§	P‡
<i>In vitro</i>	0.05 $\pm$ 0.02 (23)	0.05	0.03–0.12	—	0.021 $\pm$ 0.007 (24)	—
5 min	0.14 $\pm$ 0.09 (23)	0.13	0.04–0.36	$P_1 < 0.0001$ $P_2 = 0.02$	0.048 $\pm$ 0.020 (17)	$P_1 < 0.0001$ $P_2 = 0.008$
2 h	0.10 $\pm$ 0.05 (37)	0.09	0.03–0.20	$P_1 < 0.0001$	0.033 $\pm$ 0.015 (27)	$P_1 = 0.0004$
24 h	0.19 $\pm$ 0.14 (56)	0.17	0.04–0.80	$P_2 < 0.005$	0.045 $\pm$ 0.018 (18)	$P_2 = 0.02$
48 h	0.21 $\pm$ 0.15 (75)	0.17	0.03–0.67	$P_2 < 0.005$	0.061 $\pm$ 0.026 (21)	$P_2 = 0.0002$
7 d	0.22 $\pm$ 0.12 (73)	0.20	0.06–0.60	$P_2 < 0.005$	0.064 $\pm$ 0.025 (21)	$P_2 < 0.0001$
13–14 d	0.21 $\pm$ 0.13 (108)	0.17	0.04–0.67	$P_2 < 0.005$	0.061 $\pm$ 0.028 (86)	$P_2 < 0.0001$
28 d	0.25 $\pm$ 0.14 (107)	0.20	0.06–1.00	$P_2 < 0.005$	0.085 $\pm$ 0.026 (12)	$P_2 < 0.0001$
2 h nonmelanized	0.10 $\pm$ 0.03 (23)	0.10	0.07–0.17	—	ND	—
2 h melanized	0.16 $\pm$ 0.04 (24)	0.15	0.08–0.25	$P_3 < 0.001$	ND	—

ND, Not done.

* Mean, median and range values in μm .

† n, Number in parentheses is the number of measurements.

‡ P values calculated by *t*-test after analysis of variance. P_1 corresponds to the comparison with yeast cells fixed following *in vitro* culture in Sabouraud's dextrose broth. P_2 corresponds to the comparison versus the 2 h group, for which the inocula were grown in Sabouraud's dextrose broth. With the Bonferroni correction for multiple comparisons, significance is defined by a *P* value ≤ 0.006 . P_3 refers to comparison between cells grown in minimal medium alone (nonmelanized) and with L-dopa (melanized) studied 2 h after murine infection.

§ For some groups, the *n* differs from that used for cell wall thickness measurements as cells for which the entire cell was not visible were excluded.

mouse, except for the 13–14 d determinations, where 7–37 % of the values came from each of the four experiments. Cell wall measurements were limited to yeast cells where the sectioning occurred near the equatorial plate, as indicated by sharp cell wall edges. For intracellular localization of cryptococcal capsular polysaccharide using immunogold, immunohistochemistry was performed using mAbs 2H1 (IgG1), 12A1 (IgM) or 13F1 (IgM), murine mAbs that bind the GXM component of the polysaccharide, as described by Casadevall *et al.* (1998) and Feldmesser *et al.* (2000b). Ultrathin lung tissue sections on nickel grids were incubated in 10 % H_2O_2 for 10 min, washed in PBS, then etched in a saturated solution of sodium periodate for 10 min, washed in PBS and blocked with 2 % goat serum for 1 h. Grids were incubated overnight in 5 μg primary mAb ml^{-1} in 2 % goat serum at 4 °C. As a control, grids were incubated in murine IgG1 (Sigma) or PC-140, an IgM mAb that binds phosphorylcholine (IgM) (Thammana & Scharff, 1983). Grids were washed in PBS with 2 % goat serum with 0.1 % gelatin (60 Bloom units) and 0.01 % Tween 20 and then incubated in biotin-conjugated goat anti-mouse IgG1 or IgM (2.5 μg ml^{-1}) (Southern Biotechnology Associates) for 1 h. After washing, grids were incubated in 10 nm gold conjugated to streptavidin (Goldmark Biologicals) diluted 1:30 in 1 % bovine serum albumin for 2 h at room temperature, washed and fixed in 2 % glutaraldehyde. When comparisons were made between *C. neoformans* strains or for labelling of the same strain with different mAbs, immunolabelling of samples was performed concurrently. Labelling of all samples was performed at least twice.

Data analyses were performed using the Excel spreadsheet software package. After analysis of variance, pairwise com-

parison was performed using the Student's *t*-test. The alpha level was adjusted using the Bonferroni correction.

RESULTS

Yeast cell diameter, cell wall thickness, capsule volume and cell volume were measured at 5 min, 2 h, 24 h, 48 h, 7 d, 14 d and 28 d infection (Table 1 and Table 2). We also measured the cell wall thickness of melanized *C. neoformans* instilled into murine lungs at 2 h of infection and of *C. neoformans* cells prepared as the infecting inocula (Table 1). Pairwise comparison showed no difference in the values obtained from mice within individual experiments, except for mice studied 28 d after infection (data not shown). Both the yeast cell wall thickness and mean yeast cell diameter (excluding capsule) of cells prepared as for infecting inocula were smaller than those of yeast cells 5 min after infection. The yeast cell diameter was the same at 5 min and 2 h after infection, a finding that presumably reflects the fact that at 2 h infection, most of the yeast cells in the lung originate from the infecting inoculum. The ratio of cell wall thickness to cell diameter was lower in the infecting inoculum than at 5 min, demonstrating that the difference in cell wall thickness was not solely a function of yeast cell size. After 2 h, the mean and median cell wall thickness of *C. neoformans* increased with age of infection until the day 28 measurement. At least in part, this increase in cell wall thickness reflects an increase in

Table 2. Summary of measurements of *C. neoformans* strain 24067 cells

n refers to the number of measurements.

Condition	Total cell diameter (μm)	Total cell volume* (μm ³)	Yeast cell diameter (μm)	Yeast cell volume* (μm ³)	Capsule volume† (μm ³)
<i>In vitro</i> ‡	ND	ND	2.52 ± 0.46§ <i>n</i> = 24	9.25 ± 4.84§ <i>n</i> = 24	ND
5 min	4.51 ± 0.78 <i>n</i> = 17	52.9 ± 28.7 <i>n</i> = 17	3.07 ± 0.58 <i>n</i> = 17	16.6 ± 9.4 <i>n</i> = 17	36.2 ± 24.6 <i>n</i> = 17
2 h	5.12 ± 1.73 <i>n</i> = 10	92.9 ± 79.8 <i>n</i> = 10	3.25 ± 0.90 <i>n</i> = 10	22.0 ± 19.5 <i>n</i> = 10	70.9 ± 65 <i>n</i> = 10
24 h	9.79 ± 3.10 <i>n</i> = 16	624 ± 424§ <i>n</i> = 16	4.81 ± 1.41 <i>n</i> = 16	72.0 ± 52.6 <i>n</i> = 16	563 ± 438§ <i>n</i> = 16
48 h	11.1 ± 7.0 <i>n</i> = 19	1478 ± 1818 <i>n</i> = 23	4.26 ± 2.52 <i>n</i> = 23	87.7 ± 140 <i>n</i> = 23	1390 ± 1731 <i>n</i> = 23
7 d	4.66 ± 2.1 <i>n</i> = 20	89.7 ± 126 <i>n</i> = 20	2.67 ± 1.01 <i>n</i> = 20	14.5 ± 22 <i>n</i> = 20	75 ± 170 <i>n</i> = 20
14 d	3.59 ± 2.57 <i>n</i> = 22	259 ± 263 <i>n</i> = 22	4.25 ± 1.61 <i>n</i> = 22	60.1 ± 95 <i>n</i> = 22	198 ± 243 <i>n</i> = 22
28 d	14.0 ± 7.1 <i>n</i> = 10	2396 ± 2383§ <i>n</i> = 10	5.6 ± 2.26 <i>n</i> = 10	132 ± 119 <i>n</i> = 10	2263 ± 2282§ <i>n</i> = 10

* Volumes were calculated based upon the measured diameters.

† Capsule volume was calculated by subtracting the yeast cell volume from the total cell volume.

‡ The *in vitro* condition refers to measurements made for *C. neoformans* grown in Sabouraud's dextrose broth. Total cell measurements were not done (ND) because the capsule was not preserved following sample processing.

§ Denotes statistically significant difference from the corresponding measurement at 2 h by Student's *t*-test using the Bonferroni correction ($P < 0.007$).

Fig. 1. Cell wall thickness as a function of cryptococcal cell diameter. Each point ($n = 117$) represents one organism of strain 24067 obtained from mice infected for times ranging from 2 h through 28 d. The linear trendline demonstrates the direct relationship between cell wall thickness and cell diameter.

the size of the yeast cell diameter, as the cell wall thickness varied directly with yeast cell diameter (Fig. 1). However, to account for possible proportional increases

in cell wall thickness in relation to cell size, the cell wall diameter was also divided by the cell diameter. This ratio increased from 2 h through 28 d, and was significantly higher than the 2 h value at all times from 48 h onward. Thus, the mean cell wall thickness of *C. neoformans* increased as a function of time of infection and was not simply the result of increased cell size. The data for 13–14 d after infection shown in Table 1 were pooled from four experiments using three mouse strains. There was no difference in the values obtained from the two experiments that used C57/BL6 mice, or between A/JCr and 129/SvEv mice infected with 10^4 or 10^6 yeast cells. However, both yeast cell wall thickness and the ratio of cell wall thickness to yeast cell diameter were significantly higher in C57/BL6 mice than in A/JCr and 129/SvEv mice (data not shown). Still, comparison of data from each of these individual experiments to those from 2 h after infection showed a significant increase in cell wall thickness.

Since *C. neoformans* cells reportedly melanize during murine infection (Nosanchuk *et al.*, 1999), we hypothesized that the increase in cell wall thickness might be related to melanization. To evaluate this possibility, melanized cells obtained by growing *C. neoformans* in minimal medium with L-dopa were inoculated in the lungs and cell wall thickness was measured. The cell

Fig. 2. Appearance of *C. neoformans* strain 24067 in unstained 1 µm lung sections obtained at different times after infection, demonstrating yeast with progressively darker cell walls, which appear blacker in colour images. Panels on the right are the

wall thickness of melanized cells in lung tissue 2 h after intratracheal inoculation was significantly larger than that of cells grown in minimal medium without L-dopa. To obtain additional evidence for melanization in tissue as an explanation for cell wall thickness, we evaluated the cell wall for colour changes in unstained tissue sections by light microscopy. With increasing time after infection with nonmelanized cells, the cryptococcal cell wall became progressively blacker (Fig. 2).

In addition to changes in cell wall thickness, we noted the appearance of a heterogeneous cell population during the course of infection. A striking observation was the appearance of giant *C. neoformans* cells in infected lung tissue 24 h after infection (Fig. 3). These cells ranged up to 28 µm in diameter. Giant *C. neoformans* cells were found almost exclusively in the extracellular space. At all times of infection after 2 h, there was a wide range in the diameter of yeast cells that represented the full spectrum of sizes between the smallest and the giant forms. Similarly, increases in range of size and variance between cells were found in total yeast volume and capsule volume (Table 2). At all times of infection, there were *C. neoformans* cells with capsule dimensions corresponding to those measured at 5 min and 2 h. In contrast, examination of cells prepared as the infecting inoculum showed that of 317 yeast examined on low magnification fields ($\times 7500$), no yeast had a cell diameter > 3.3 µm (range: 1.1–3.3 µm). Thus, the appearance of giant cells was not the result of size heterogeneity of the infecting inoculum. These observations indicate that *C. neoformans* pulmonary infection in mice is associated with the emergence of heterogeneous yeast cell populations remarkable for a range of cell and capsule size.

To establish whether the observations made with strain 24067 were applicable to serotype A strains, mice were infected with strain H99 and lung sections were studied at 2 h and 14 d after infection. For strain H99, the mean cell wall thickness did not significantly increase during infection (mean cell wall thickness at 2 h was 0.20 ± 0.04 vs 0.31 ± 0.3 µm at 14 d; $P = 0.269$; $n = 12$ for 2 h, $n = 48$ for 14 d measurement). However, unlike strain 24067, which produced yeast cells representing the continuum of cell size between smaller and giant forms, for strains H99 and a second serotype D strain (3501), two more discrete populations emerged that could be divided into two groups – giant and micro forms. Examination of 1 µm toluidine-blue-stained sections demonstrated that at 14 d after infection, the mean percentage of yeast that were giant forms was $69.7 \pm 20.3\%$ and $70.5 \pm 27.4\%$ for strains H99 and 3501, respectively (for H99, 20 fields were counted at a magnification of $\times 200$; for 3501, 13 fields were counted). Because the cell wall thickness of many micro forms of strain H99 was smaller than that

phase-contrast images of the panels on the left, which were imaged using standard optics. Times after infection: (a) 24 h; (b) 48 h; (c) 7 d; (d) 14 d; (e) 28 d. Bars, 10 µm.

Fig. 3. Giant and smaller *C. neoformans* cells coexist in tissue. (a) Strain 3501, 24 h after infection. (b) Strain 24067, 48 h after infection. Bars, 10 μ m. Arrows point to smaller intracellular yeast.

Fig. 4. Intracellular location of epitope for the capsular polysaccharide. Immunogold labelling with mAb 12A1 demonstrated labelling of the cell wall and cytoplasmic vesicles in addition to the capsule. (a, b) Micrographs shown are from mice infected for 48 h with *C. neoformans* strain 24067. Cap, capsule; CW, cell wall. Arrows point to cytoplasmic vesicles containing gold particles. Bar, 1 μ m.

seen at 2 h, measurement of the two populations yielded a mean cell wall thickness that was not significantly different.

The availability of tissue sections from different times of infection allowed us to investigate whether the epitope recognized by protective antibodies was expressed at all

times of infection. Labelling of cells from tissue obtained from mice infected from 2 h through 28 d demonstrated the presence of gold particles in all three locations. Unexpectedly, this study also provided information on the site of capsule synthesis, since it appeared that in some yeast, there was intracellular immunogold staining in addition to that found in the capsule structure, as expected. To study the location of capsule synthesis, immunogold labelling with mAb 2H1 was performed on lung tissue obtained from mice infected with strain 24067 for times ranging from 2 h through 28 d. IEM demonstrated the presence of the epitope not only in the capsule, but also in the cryptococcal cell wall and cytoplasm (Fig. 4), where it appeared to be localized primarily to membrane-bound vacuolar structures. For tissue obtained from mice infected for 48 h, the same pattern of labelling was seen when the IgM mAbs 12A and 13F1 were used. For strain 3501, labelling of tissue from mice infected for 24 h with mAb 13F1 produced a pattern and intensity of labelling comparable to that seen for strain 24067 (Fig. 5). However, both mAbs 2H1 and 12A1 labelled both the capsule and intracellular locations of strain 3501 less intensely than for strain 24067. To determine whether the epitope was present in acapsular cells, immunogold labelling of Cap 67 was performed. For Cap 67, only occasional gold particles were present when mAbs 2H1 or 12A1, which bind the same epitope, were used. However, for mAb 13F1, gold label was present in the cell wall. Very rare gold particles were present on sections from all three strains labelled with the control IgG or PC-140 Abs, or on sections of normal lung from mice infected for 5 min labelled with mAb 13F1, indicating that labelling was specific.

DISCUSSION

The goal of this study was to establish whether the characteristics of yeast cells changed during the course of murine *C. neoformans* pulmonary infection and to identify yeast cellular characteristics that may provide insight into the mechanism by which *C. neoformans* establishes chronic infections that are ultimately lethal to mice. The results reveal a highly dynamic process whereby major changes occur in *C. neoformans* cells during the course of experimental murine infection. Morphological changes are potentially relevant to cryptococcal pathogenesis because they suggest the possibility that, *in vivo*, new cellular forms arise from selection and/or changes stimulated by the growth in tissue conditions. The generation of new variants that present different problems to host immune effector cells may contribute to persistent infection.

Cell wall thickening occurred early in the course of infection and was maintained during chronic infection. These results confirm the work of Sakaguchi *et al.* (1993), who reported that the cell wall of *C. neoformans* in tissue is thicker than that of cells grown *in vitro*. However, we extended that finding by showing that the increase in cell wall thickness is maintained following normalization for changes in cell diameter, thus rigorously demonstrating a progressive thickening of the

cell wall during infection. We hypothesized that a mechanism for the increased cell wall thickness was melanization *in vivo*, given recent evidence that *C. neoformans* cells synthesize melanin during infection (Nosanchuk *et al.*, 1999). To evaluate this possibility, we compared the cell wall thickness of melanized and nonmelanized cells. *C. neoformans* cells grown in medium with L-dopa had significantly thicker cell walls than did nonmelanized cells, demonstrating that melanization increases cell wall thickness. The finding that melanization of *C. neoformans* cells *in vivo* is progressive and takes several days is consistent with the timing of the increase in cell wall thickness observed in this study. Since there has been some controversy regarding the extent of *in vivo* melanization (Liu *et al.*, 1999), we sought to obtain additional evidence that this process was in fact occurring in our system. Light microscopic analysis of unstained tissue sections revealed that cryptococcal cell walls became progressively darker during the course of infection, a finding that we attribute to melanin formation. Though melanin in the cryptococcal cell wall is probably amorphous (Nosanchuk *et al.*, 1999), the presence of this additional material may cause the observed increased cell wall thickness. A melanized cell wall may serve a protective role by shielding the yeast cell from host antimicrobial substances (Wang & Casadevall, 1994).

In evaluating cell wall thickness, we considered the sources of error that can impact on measurement. The first potential source of error results from measurement of sectioned images of cells that are approximately spherical, as measurements of cell wall thickness in cells sectioned near the pole would yield larger values than measurements of cells sectioned near the equator. Therefore, we only measured cells with cell walls that both appeared sharp and had relatively constant thickness throughout the circumference of the cell. We avoided cells with blurred edges, which arise from sectioning near the pole, and those with varying thickness, which result from sectioning on a plane that is not perpendicular to the cross-section of the cell. The second potential source of error comes from averaging the size and shape of a cell population that is not normally distributed. For strain 24067, cell size appeared to be continuous, whereas for H99, there clearly were two populations of cells that differed sharply in size. For H99, averaging the cell walls of all cells imaged did not result in a statistically significant increase in size because of the increased variance introduced by measurements of giant cells. A third potential source of error is cell wall changes introduced by fixation, staining and processing of tissue. We noted that the cell wall thickness changed when comparing cells in culture to those from tissue 5 min after infection. The ratio of cell wall thickness to cell diameter in the infecting inoculum was the same as that at 2 h, a finding that may reflect osmotic differences between culture conditions and the lung, or an initial rapid alteration in yeast in response to infection. Alternatively, the difference in cell wall thickness noted between cells fixed from *in vitro* culture and from tissue

Fig. 5. For legend see facing page.

5 min after infection may reflect improved preservation of the cell wall structure in lung tissue in a manner similar to that seen for capsule architecture (Feldmesser *et al.*, 2000b). Regardless of the explanation, this difference in cell wall thickness between *in vitro* yeast cells and those in tissue obtained 5 min after infection does not affect the conclusions of this study because the relevant comparisons all involved measurements of cells in tissue. Furthermore, all samples were processed in an identical fashion and any errors or artefacts introduced by the sample preparation method should apply equally to all groups being compared. We found little variability in the measurements made between mice within individual experiments, with the exception of C57BL/6 mice studied 28 d after infection, a time at which these mice have developed a granulomatous immune response. The finding of variability late, but not early, after infection may reflect individual differences in the degree to which mice can control this organism. Further, in tissue obtained 2 h after infection, there was no difference in the measurements of yeast cells made in C57/BL6 or BALB/c mice. However, a difference was seen in sections obtained from different mouse strains on day 13 or 14 after infection. Since both A/JCr and 129/SvEv mice are more susceptible to pulmonary cryptococcal infection than are C57BL/6 mice (Feldmesser *et al.*, 1998) (personal observation), more replication of yeast in these mouse strains may result in a relative preponderance of younger yeast cells with thinner cell walls. However, definitive correlation of cell wall thickness with host inflammatory response would require further investigation and is beyond the scope of the present study. We are confident that for strain 24067, the mean cell wall size increases during infection but the generalizability of this observation to other cryptococcal strains is unknown.

The morphology of *C. neoformans* cells in infected tissue was more heterogeneous than when grown *in vitro*. Heterogeneity in the morphology of *C. neoformans* cells involved both capsule size and yeast cell size. The emergence of giant cells was a striking observation. These cells, found predominantly in extracellular spaces, were much larger than tissue macrophages. In contrast, cells with smaller capsules were commonly found inside macrophages in well-defined vacuoles. Presumably, the emergence of giant cell forms poses a formidable problem for phagocytic cells since their large size relative to macrophages would preclude ingestion. Giant cryptococcal forms have been reported from two cases of human disease, one isolated from the lung and the other from cerebrospinal fluid (Cruickshank *et al.*, 1973; Love *et al.*, 1985). The paucity of such reports in the literature could result from the lack of histopathological data from primary pulmonary infection in humans, which is seldom symptomatic. However, that such isolates have been described suggests

that giant forms have relevance to the pathogenesis of human disease. Morphologic variation within lesions has been described for *Paracoccidioides brasiliensis* and *Histoplasma capsulatum* (Restrepo, 2000; Sweany *et al.*, 1962). The mechanism responsible for the generation of this diversity in cellular morphology is not understood, but may involve phenotypic switching, phase growth differences, and/or nutrient differences between *in vivo* and *in vitro* conditions (Goldman *et al.*, 1998). The pattern of heterogeneity in yeast cell size varied between *C. neoformans* strains. Although the mean capsule size increased significantly during the course of infection, not all cells displayed larger capsules and populations of cells with large and small capsules coexisted in tissue. Heterogeneity occurred early in the course of infection and was maintained at all times studied. The emergence of giant forms and the heterogeneity of cell size indicate that the immune system must confront cells with varying characteristics during the course of infection. This variation could contribute to the difficulty inherent in controlling this infection.

Given the present efforts to develop antibody therapy for human cryptococcosis and the variability inherent in *C. neoformans* cells from chronically infected mice, we evaluated whether the epitope recognized by the protective mAb 2H1 was found at all stages of infection. mAb 2H1 bound to the capsule of yeast cells at all times of infection, implying that epitope loss is not a consequence of yeast cell heterogeneity. During the course of this study, we noted that some antibody staining occurred intracellularly. Since very little is known about the location of capsule synthesis in *C. neoformans* (Doering, 2000), we investigated the location of mAb-reactive epitope in the yeast cell. Though previous studies by conventional freeze-etching (Takeo *et al.*, 1973) and quick freeze-deep etching methods (Sakaguchi *et al.*, 1993) have suggested that precursors are synthesized in cytoplasmic vacuoles or in the particle-accumulating layer of the cell wall, no definitive evidence associates the capsule synthesis machinery with the cell wall. In this study, immunogold labelling with mAb to GXM demonstrated the presence of the epitope not only in the capsule, but also in the cryptococcal cell wall and cytoplasm, where it appeared to be localized primarily to membrane-bound vacuolar structures. Capsule components containing the epitope recognized by mAb 2H1 may be synthesized intracellularly and exported through the cell wall, possibly in the small vesicles described by Sakaguchi *et al.* (1993). Further confirmation of this finding will require the identification of markers for cryptococcal vacuoles. These results provide the first direct evidence that capsule synthesis occurs, at least in part, intracellularly.

Among the two serotype D strains (24067 and 3501) studied for mAb binding, we noted qualitative and quantitative differences in the antibody binding pattern.

Fig. 5. Immunogold labelling of strains Cap 67 and 3501 from murine lung 24 h after infection. (a, b) Strain Cap 67 incubated with PC-140 (IgM control) (a) or mAb 13F1 (b); (c–f) strain 3501 incubated with IgG1 (c), mAb 2H1 (d), mAb PC-140 (e) or mAb 13F1 (f). Only rare gold particles are present in control sections (a, c, e). Bar, 1 µm.

We attribute this finding to subtle differences in GXM structure between strains. Previous studies of yeast cells grown *in vitro* have shown both significant differences in capsular polysaccharide within a serotype (Small *et al.*, 1986) and heterogeneity within serotypes in the expression of epitopes reactive with mAbs (Spiropulu *et al.*, 1989). GXM structure is notoriously variable among strains and even individual strains can produce different types of GXM depending on the phenotype (Fries *et al.*, 1999).

We unexpectedly found that the cell wall of Cap 67 labelled with mAb 13F1, though not mAbs 2H1 or 12A1. All three mAbs were generated from the spleen of the same GXM-tetanus toxoid immunized mouse. mAbs 12A1 and 13F1 were derived from the same B cell clone (Mukherjee *et al.*, 1993), but differ in fine specificity, binding pattern on intact yeast cells, and protective efficacy (Mukherjee *et al.*, 1993, 1995). Though the mechanisms and genes involved in capsular synthesis are current areas of study for several investigators (Chang *et al.*, 1996; Doering, 1999), the molecular basis for the absence of capsule in this mutant is unknown (Fromtling *et al.*, 1982; Jacobson *et al.*, 1982). The present study shows that the epitope recognized by mAb 13F1 is produced by this mutant, though labelling was less intense than in its isogenic parent strain. The defect in this mutant may lie, in part, in its ability to export this polysaccharide component through the cell wall, though a little label was found in adjacent tissue, suggesting that this is not the case. However, the epitope recognized by mAbs 2H1 and 12A1, which have similar, if not identical, fine specificity, was not found in Cap 67, suggesting that synthesis of the component of GXM that forms the epitope for these mAbs is defective. This result raises the tantalizing possibility that the epitopes recognized by the mAbs 12A1 and 13F1 reside in different GXM molecules, a finding that would imply that more than one type of GXM molecule is produced by some strains of *C. neoformans*.

In summary, our study demonstrates that murine *C. neoformans* infection is a highly dynamic process whereby the morphological characteristics of yeast cells differ as a function of the age of infection. We noted significant differences between yeast cells studied after growth *in vitro* and the population of yeast cells that emerges in tissue during chronic infection. Although it has been recognized for several decades that infection results in morphological changes highlighted by increased capsule size and cell wall thickness, the occurrence of other changes in cellular characteristics is not widely recognized.

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