# Urease Expression by *Cryptococcus neoformans* Promotes Microvascular Sequestration, Thereby Enhancing Central Nervous System Invasion

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**Our objective was to determine the role of the cryptococcal virulence factor urease in pulmonary-to-central nervous system, dissemination, invasion, and growth. *C. neoformans* H99, the urease knockout strain (*ure1*) derived from H99, and the urease restored strain *ure1+URE1−1* were used for the studies. The absence of cryptococcal urease (*ure1* infection) resulted in significant protection from the high mortality observed in H99-infected mice. All H99-infected mice had extremely high cryptococcal loads in their brains at the time of death, whereas only two of six animals that died of *ure1* infection had detectable *C. neoformans* in the brain. Histological analysis of the blood-to-brain invasion by *C. neoformans* H99 demonstrated wedging of the yeasts in small capillaries, altered structure of microvessel walls, formation of mucoid cysts initiated in the proximity of damaged microcapillaries, and the absence of an inflammatory response. Direct inoculation of H99, *ure1*, and *ure1+URE1−1* into the brain demonstrated that urease was not required to grow in the brain. However, the dissemination patterns in the brain, spleen, and other organs after intravenous inoculation indicated that cryptococcal urease contributes to the central nervous system invasion by enhancing yeast sequestration within microcapillary beds (such as within the brain) during hematogenous spread, thereby facilitating blood-to-brain invasion by *C. neoformans*. (Am J Pathol 2004, 164:1761–1771)**

Dissemination into the central nervous system (CNS) and the subsequent development of meningitis/encephalitis is a major cause of mortality during infections with oppor- tunistic yeast *Cryptococcus neoformans*.[1–3] Although high CNS tropism characterizes *C. neoformans*; the cryptococ- cal virulence factors that contribute to this high CNS invasiveness are largely unknown.[4–6] So far only lac- case, an enzyme catalyzing cryptococcal melanin pro- duction, has been demonstrated to be critical for the survival of *C. neoformans* in the brain.[7,8] As more data about cryptococcal virulence factors becomes available, it is also becoming apparent that a combination of viru- lence factors rather than a single virulence factor is most likely involved in multistep process of cryptococcal dis- semination and CNS invasion.[7,9–13] However, under- standing how *C. neoformans* transfers from the primary sites of infection into the CNS is incomplete. The steps in pulmonary-to-CNS dissemination include escape out of the primary infection site, fungemia, and transport through the blood, transfer from the blood into the CNS, and colonization/growth in the CNS.[7,14–16] The most fre- quently proposed mechanism of cryptococcal CNS inva- sion involves the initial transfer of circulating cryptococci into the cerebrospinal fluid/subarachnoid space and the development of leptomeningitis. Subsequent progression of the infection from the brain surface into the cortex, and cryptococcal growth along the Virchow-Robin spaces into the deeper parts of the brain is thought to lead to fatal brain infection. Alternatively, it has been proposed that *C. neoformans* can directly cross the blood brain barrier at the level of small cerebral capillaries. This suggestion was based on the observation that in some cases cryp- tococcal cysts develop within the brain in the absence of meningitis (parenchymal form of cryptococcal meningo- encephalitis)[3] and the finding of isolated cryptococcal lesions in the proximity of small microvessels in necropsy studies.[1] On the cellular level, a transfer across the en- dothelial barrier could occur via direct extravasation, en- dothelial transcytosis,[17] or with help of mononuclear

phagocytes serving as Trojan horses.[15] Even though a direct evidence of *C. neoformans* transfer into the CNS has not been presented to date, it is possible that more than one mechanism (route) can lead *C. neoformans* into the CNS and in each case, different virulence factors may be important.

Urease was recently identified as an important virulence factor of *C. neoformans*.[18] Although urease-negative isolates have been sporadically reported,[19–21] the overwhelming majority of *C. neoformans* strains produce this extracellular enzyme and invest a significant amount of energy in urease production.[19] The mechanism by which urease could mediate the virulence in opportunistic yeasts is unknown. To study the role of cryptococcal urease, a urease knockout strain (*ure1*) was produced by targeted deletion of urease gene from H99.[18] Strains H99 and the newly developed *ure1* and the *ure1+URE1−1* (strain *ure1−* rescued with a wild-type copy of URE1 gene) provide a valuable tool to study the role of urease as a cryptococcal virulence factor. Using these strains, it has been demonstrated that urease expression by *C. neoformans* has a detrimental effect on the survival of mice infected via the intravenous or intranasal route.[18] The mechanism by which cryptococcal urease contributes to the high mortality is unknown, but mortality in both clinical and experimental cryptococcosis primarily correlates with the invasion of the CNS.[22–26] When cryptococci were directly injected into the cerebrospinal fluid of corticosteroid-pretreated rabbits, urease expression did not affect growth in the CNS.[18] This result suggested that urease was not required for the optimal growth of *C. neoformans* in the CNS; however, it did not negate the possible role for urease in the dissemination process. Our objective was to determine the role of cryptococcal urease in pulmonary-to-CNS dissemination, invasion, and growth.

## Materials and Methods

### Cultures of C. neoformans

For this study, the wild-type strain H99 and two genetically modified derivatives were used: *ure1* (urease-negative transformant of H99 with a selective disruption of native urease gene, *URE1*), and *ure1+URE1−1* (strain *ure1−* rescued with a wild-type copy of URE1 gene). H99 is a widely studied clinical isolate of serotype A. This strain is virulent in immunocompetent mice and is characterized by capsule, laccase/melanin, phospholipase B, and urease activity.[9,18,27–29] The development of *ure1* and *ure1+URE1−1* strains was previously described.[18] For infection, yeasts recovered from frozen 10% glycerol stocks were grown to stationary phase (at least 72 hours) in Sabouraud dextrose broth (1% neopeptone, 2% dextrose; Difco, Detroit, MI) at 36°C on a shaker. The cultures were then washed in nonpyrogenic saline (Travenol, Deerfield, IL), counted on a hemocytometer, and diluted to the required concentrations in sterile nonpyrogenic saline.

### Mice

Mice (C57BL/6×129F2; Jackson Laboratories, Bar Harbor, ME) were selected to study the effects of cryptococcal urease because they are able to clear the low-mid virulent strains of *C. neoformans* but succumb to the highly virulent ones.[25,30,31] These mice were housed under specific pathogen-free conditions in enclosed filter top cages at the University of Michigan Unit for Laboratory Animal Medicine. Clean food and water were given *ad libitum*. The mice were handled and maintained using microisolator techniques with daily veterinarian monitoring. This study was approved and monitored by the University Committee on the Use and Care of Animals at the University of Michigan.

### Infection with C. neoformans

For the intratracheal and intracranial infections mice were anesthetized by intraperitoneal injection of ketamine/xylazine mix (ketamine/xylazine, 100/6.8 mg/kg/body weight) and restrained on a foam plate. For the intratracheal infection, trachea was exposed by small incision through the skin and separation of underlying tissue. A bent 30-gauge needle (Becton Dickinson, Rutherford, NJ) attached to a tuberculin syringe (BD&Co., Franklin Lakes, NJ) with the adjusted *C. neoformans* culture was inserted into the trachea. Inoculum ($10^4$ yeast cells in 30 $\mu$l) was dispensed into the lungs. The skin was closed with cyanoacrylate adhesive. The mice recovered with minimal visible trauma. The intracranial infection was performed as described elsewhere.[32,33] Briefly, the inoculum ($10^4$ yeast cells in 20 $\mu$l) was injected into the central occipital area of a cranium via a 27-gauge needle inserted as shallowly as possible (less than 5 mm). The intravenous infection ($10^5$ or $10^6$ yeast cells in 0.25 ml of nonpyrogenic saline) was performed by the tail vein injection of unanesthetized animals. Aliquots of the inoculums were collected and plated on Sabouraud dextrose agar (Difco) to monitor the number of colony forming units (CFU) being delivered. The actual depositions for the different procedures are included in the figure legends. No significant differences between inoculi sizes between three strains at each experiment were observed.

### Organ and Blood CFU Assays

At selected time points, mice were humanely euthanized by $CO_2$ asphyxiation, lungs, spleens, kidneys, hearts, and brains were dissected and homogenized in 2 ml of sterile water and serially diluted in sterile water. Blood was collected aseptically via cardiac puncture immediately after euthanasia. Dilutions of organ and blood samples (10 $\mu$l each) were plated on Christensen's urea agar (containing 300 nm of urea and phenol red as a pH indicator) and incubated at room temperature for 48 hours. Colony counts were performed and adjusted to reflect the total CFU/organ. Media color change in proximity of colonies confirmed the urease activity in H99 and

*ure1+URE1−1*, and the absence of urease activity in *ure1*, isolated from organs of infected animals.

## Serum Incubation CFU Assay

Samples of H99, *ure1*, and *ure1 +URE1−1* cultures prepared for intravenous injection were prepared in nonpyrogenic saline at a concentration of $10^6$ cells/ml. These microbial suspensions (10 $\mu$l) were combined with freshly isolated mouse serum (90 $\mu$l) and incubated for 3 hours on 96-well plate in triplicates for each strain. At the same time $10^4$ cells of each strain were incubated in 100 $\mu$l of Saburaud dextrose broth (SDB) as the control. After the incubation samples were serially diluted in sterile water. Dilution samples (10 $\mu$l each) were plated on Sabouraud dextrose agar and incubated at room temperature for 48 hours. Colony counts were performed and expressed in log CFU/well.

## Peritoneal Macrophage Phagocytosis Assay

Peritoneal macrophages were washed from the peritoneal cavities of uninfected mice, washed, adjusted to $10^6$ cells/ml, and allowed to adhere to the bottom of the Lab-Tek II chamber slides (Nalge Nunc Int., Rochester, NY) for 1 hour. H99, *ure-1*, or *ure1+URE-1−1* organisms preopsonized in mouse serum were added to different chambers (10:1 cell/target ratio) in duplicates. After 3 hours of incubation (37°C, 5% $CO_2$) slides were washed with phosphate-buffered saline to remove free-floating yeast cells, fixed, and stained with Diff-Quick (Dade Behring Inc., Newark, DE). Numbers of ingested yeast were evaluated in 200 macrophages for each culture.

## Histology

Lungs were fixed by inflation with 1 ml of 10% neutral buffered formalin, excised *en bloc*, and immersed in neutral buffered formalin. Brains were carefully excised and immersed in neutral buffered formalin. After tissue processing and paraffin embedding, 2- to 3-$\mu$m sections of both organs were cut and stained with mucicarmine and hematoxylin and eosin. Up to 20 consecutive sections of the brain were prepared for examination and analyzed. Sections were analyzed with light microscopy at low and high power to evaluate cryptococcal invasion and size/location of lesions in the brain. Size of the lesions and their associations with anatomical structures was determined by examination of the same lesion on adjacent consecutive sections.

## Calculations and Statistics

Data (mean ± SE) for each experimental group were derived from at least two independent infections and analyzed via *t*-test, one-way or two-way analysis of variance, depending on the number of groups. For individual comparisons of multiple groups, post hoc Tukey's test was used to calculate *P* values. Survival between the groups was compared using Kaplan-Meier survival analysis coupled with log rank test. Means with $P < 0.05$ were considered statistically significant.
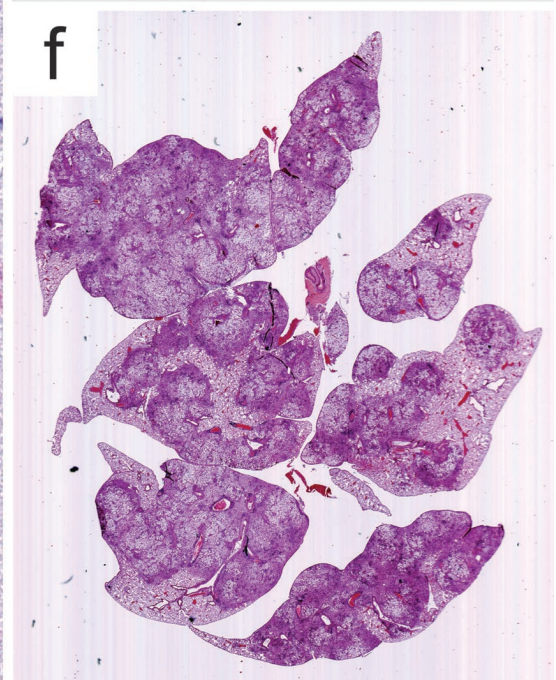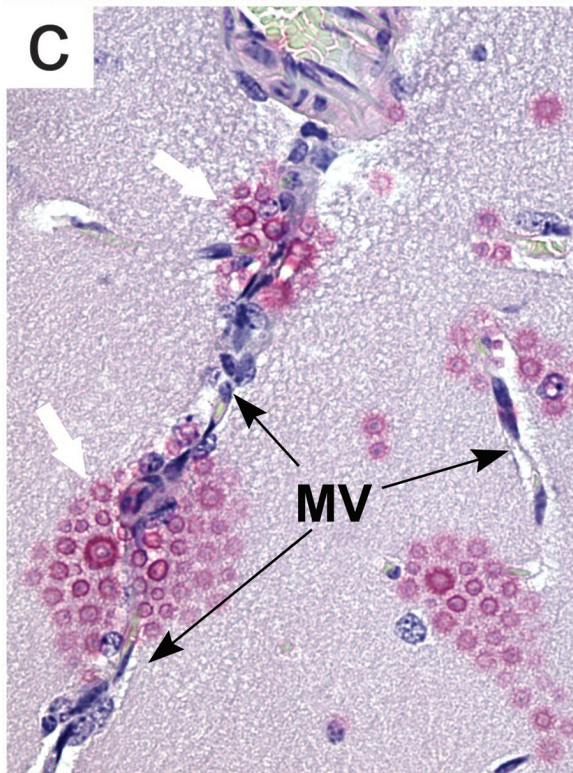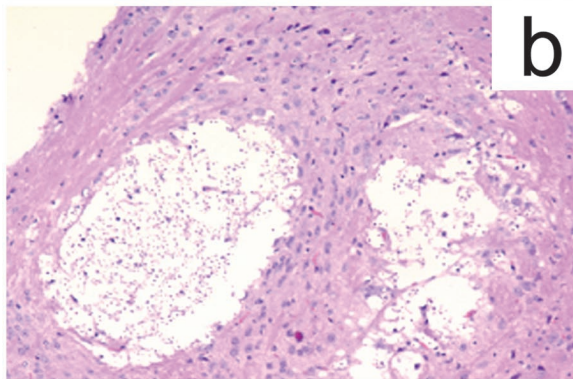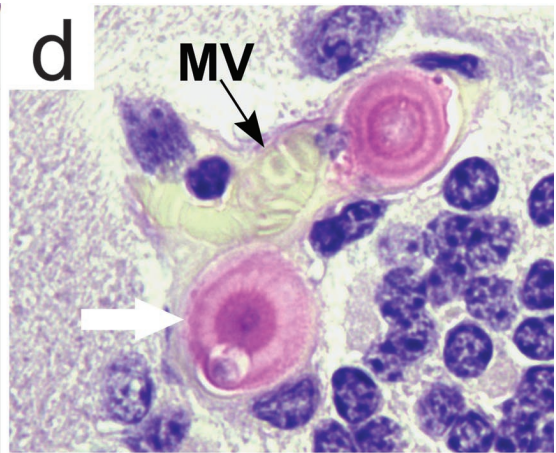
## Results

### Lung-to-Brain Dissemination, CNS Growth, and Neurological Symptoms Accompany the High Mortality in Mice Infected Intratracheally with Urease-Producing C. neoformans *H99*

Intratracheal administration of urease-producing *C. neoformans* H99 resulted in uniform mortality of C57BL/6 × 129F2 mice: 75% of the H99-infected mice died by 4 weeks of infection and 100% of animals died by week 6 of infection. To determine whether the high mortality rate was accompanied by CNS invasion, we monitored moribund animals for the presence of neurological symptoms and performed pathological examination of the brains. Before death, we observed neurological symptoms such as extension of the cerebral portion of cranium, abnormal gait, paralysis, and lethargy. Histological analysis of brains postmortem demonstrated multiple cysts up to 1.5 mm in diameter containing *C. neoformans* (Figure 1, a and b). This severe brain pathology observed in H99 infection was consistent with neurological symptoms and high mortality because of massive disseminated growth of cryptococci in the brains of infected mice.

### Mechanism of C. neoformans *Entry into the Brain—CNS Dissemination after Pulmonary Infection with H99*

To determine how *C. neoformans* H99 enters the brain parenchyma during pulmonary-to-CNS dissemination in mice, we performed extensive histological analysis of consecutive brain sections in mice 40 days after intratracheal infection (pulmonary/natural route) as well as 20, 40, and 72 hours after intravenous inoculation. After intratracheal and intravenous inoculations, the cysts in the brains were observed in diverse areas of the brain, including brain stem, colliculus, dentate gyrus, amygdala, cerebellum, and lateral cortex/Ammon horns but not within the leptomeninx. Mucoid cysts were entirely embedded within the brain parenchyma and had no contact with the leptomeningeal surface (determined by analysis of serial brain sections). The newly formed microcysts were located in direct proximity of the microcapillaries (Figure 1c), whereas intravascular cryptococci were found individually or in small clusters (Figure 1d and Figure 2, a and b) frequently in different stages of cell division/budding (Figure 1d and Figure 2; a to c). Growing cryptococci formed a contact with the endothelial surface, and appeared to distend the capillaries (Figure 1d). In some areas the small vessels were completely occluded by intravascular cryptococci as indicated by lack of blood cells in these microvessels (Figure 2c). In both partially and completely occluded microvessels, en-

dothelial cells appeared compressed (Figure 1d; Figure 2, b and c) and their structure was blurred (Figure 2, c and d) and in some areas the capillary wall was no longer visible under the light microscopy (Figure 1d; Figure 2, a and d). Cryptococcal growth into the brain parenchyma progressed from the perivascular space of these occluded microvessels into the deeper layers of brain tissue, suggesting that *C. neoformans* H99 invaded brain parenchyma via direct transfer across the blood/brain barrier at the sites were cryptococci embolized microcapillaries (Figure 2; a to c). Both intravascular and extravascular growth of *C. neoformans* occurred without local leukocyte accumulation. Sporadically, individual neutrophils could be found in the proximity of intravascular yeast cells (Figure 1d), however no evidence of intravascular or interstitial granuloma formation could be found at any level of the lesion. The halos of mucopolysaccharide were invisible or minimal around the intravascular yeasts (Figure 1d; Figure 2, a and d) but it became apparent within occluded portions of microvessels (Figure 2c), and within neighboring brain parenchyma (Figure 2; b, c, and e). In all individuals studied, no visible invasion of subarachnoid space was visible. Leptomeningeal surfaces, cerebrospinal fluid in brain ventricles, and Virchow-Robin spaces along the blood vessel entering the brain from its surface and plexus choroids were free of cryptococci, regardless of the widespread infection in the brain parenchyma (data not shown). On the intravenous injection, formation of small mucoid microcysts was observed as early as 20 hours after infection, however multiple microcysts were evident at 36 hours after infection (Figure 2, d and e) indicating that the transfer from blood into the brain parenchyma may occur as early as 20 hours, and clearly before 36 hours from the onset of H99-fungemia. These primary microcysts resembled the small, presumably newly developed cysts that were found in the brains of intratracheal-infected animals (Figure 2; a to c). In summary, invasion of the CNS by *C. neoformans* H99 occurs in central parts of the brain in the proximity of microvessels occluded by cryptococci rather than from the surface of the brain via the invasion of leptomeninx.

## Effect of Cryptococcal Urease Gene Deletion on Extrapulmonary Dissemination and Host Survival after Intratracheal Inoculation

Our next objective was to determine the contribution of cryptococcal urease to the virulence of *C. neoformans* H99 in terms of survival and CNS invasion after pulmonary infection in C57BL/6×129F2 mice. Mice were infected intratracheally with H99 ($n = 20$) or *ure1* ($n = 21$).

Cumulative survival and brain pathologies were compared. By week 4 of infection, no mortality had occurred in mice infected with *ure1*. In contrast, there was 75% mortality in the H99-infected group by week 4. Only 30% of *ure1*-infected mice died during the 8-week course of infection whereas all of the H99-infected animals died by week 6. Neurological symptoms were not apparent in mice that died from *ure1*; however, these mice demonstrated a rapid shallow breathing pattern before death. Postmortem analysis of this group of 30% of *ure1*-infected mice (weeks 6 to 8 after infection, $n = 3$ for histology) indicated that these mice had no visible evidence of damage in the brain (Figure 1e). However, multiple solidified nodules, defined histologically as granulomas, encompassed almost the whole lungs (Figure 1f). The central portion of these granulomas contained dividing cryptococci with large capsules, surrounded by mixed leukocyte infiltrate. No macroscopic abnormalities were observed in other internal organs. Thus, deletion of the urease gene from *C. neoformans* prevented long-term cryptococcal infection/pathology in the brain and prolonged host survival after pulmonary inoculation with the fungus.

Next, we compared the cryptococcal loads in the lungs and brain at time of the death in H99 *versus ure1* infections to determine whether the differences in survival and CNS pathology were associated with differences in pulmonary clearance. Total lung CFU analysis at the time of death revealed high cryptococcal burdens in *ure1* infection (7.95 ± 0.70 log CFU, weeks 6 to 7) that was similar to those observed in H99-infected lungs at the time of death (8.70 ± 0.30 log CFU, weeks 3 to 6). These data indicated that longer survival and minimal brain pathology in *ure1*-infected mice was not because of clearance of the infection from the primary site of infection (the lungs). All mice that died from H99 infection had extremely high cryptococcal loads in their brains (Figure 3A), whereas only two of the six animals that died of *ure1* infection cultured positive for *C. neoformans* in the brain (Figure 3A, limit of detection = 100 CFU). At week 10 after infection, all of the surviving *ure1*-infected mice were euthanized and the brain CFUs were analyzed (Figure 3B). At week 10, brain CFU was detected only in 33% of the surviving *ure1*-infected mice. Collectively, these data indicate that nonsurviving *ure1*-infected mice did not die from CNS infection, but most likely they died from ongoing pulmonary infection.

We next assessed whether urease expression facilitated earlier dissemination of *C. neoformans* from the lung into the blood/spleen and brain. Cryptococcal burdens in brains and spleens (index of hematogenous dissemination from the lung) were assessed on day 16 after infec-

**Figure 1.** Effect of *C. neoformans* urease on the CNS pathology. Mice were infected intratracheally with 3.87 log CFU of *C. neoformans* H99 (**a–c**) or 4.00 ± 0.09 log CFU of *C. neoformans ure-1* (**d** and **e**). Brains and lungs of mice that succumbed to these infections were analyzed by light microscopy of sections stained with combined Mucicarmine/H&E stains. **a:** Cryptococcal cysts located in central nuclei colliculi (bilateral) and in cerebellum 4 weeks after infection with H99. **b:** Expanding cryptococcal mucoid cysts infiltrating and displacing the brain tissue. Note the absence of inflammatory infiltrate. **c:** Localization of *C. neoformans* (**white arrows**) growth sites in the brain parenchyma adjacent to brain microvessels (MV, **black arrows**). **d:** Intravascular cryptococci growing/budding in a thin-walled capillary of cerebellum. Note the close contact of yeast cells with the endothelium, distended vessel wall, and flattened endothelial cells in proximity of yeast cell. **e:** Brain without the evidence of CNS infection 8 weeks after intratracheal infection with *ure-1*. **f:** Pulmonary infection. Note the extensive lung damage and demarcation at the edge of granulomas. Original magnifications: ×6 (**a, f**); ×33 (**b**); ×132 (**c**); ×330 (**d**).
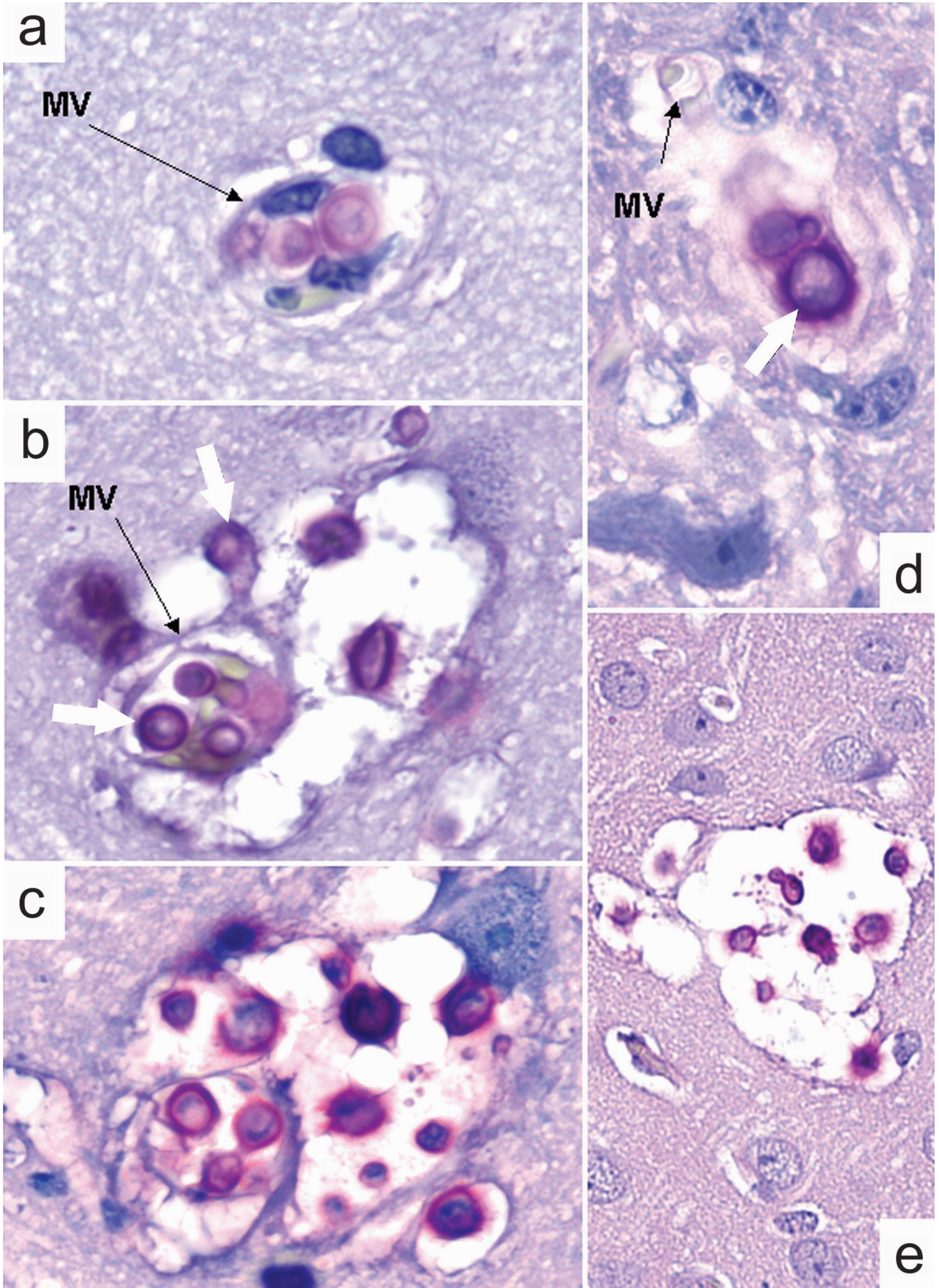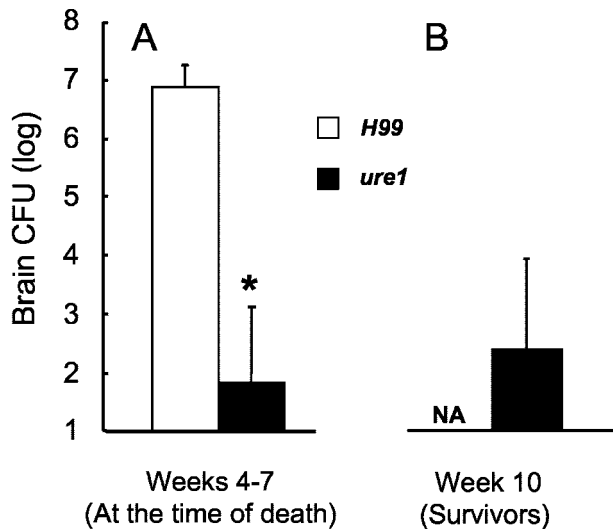
**Figure 3.** Effect of *C. neoformans* urease on brain CFU loads at the time of death after the intratracheal infection with $4.00 \pm 0.09$ log CFU (**A**) and brain CFU in mice surviving the infection with *ure1* 10 weeks after infection (**B**). Data, pooled from separate matched experiments, are expressed as the mean CFU per brain $\pm$ SE; *, $P < 0.05$ in comparison with H99 infection, $n = 5$ to 18 per group.
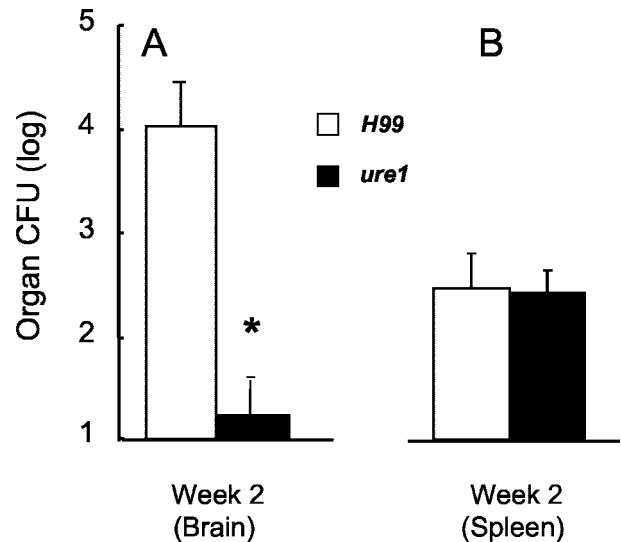


**Figure 4.** Role of *C. neoformans* urease in systemic dissemination of *C. neoformans* from the lung. Bars represent CFU organ load at 2 weeks after infection with $4.14 \pm 0.07$ log CFU intratracheally found in the brain (**A**) and spleen (**B**). Data, pooled from three separate matched experiments, are expressed as the mean CFU per organ $\pm$ SE; *, $P < 0.05$ in comparison with H99, $n = 10$ to 18 per group.

tion (a week before any deaths of infected animals). Mice infected with the urease producing strain H99 already had a 1000-fold higher cryptococcal burden in the brain compared to *ure1*-infected mice (Figure 4A). The frequency of CNS colonization was 94% in H99-infection compared to only 40% of mice infected with *ure1*. In contrast with the large differences in brain colonization and CFU, the frequency of spleen colonization and distribution of individual spleen CFU was not different between H99- and *ure1*-infected mice (Figure 4B). Thus, urease did not appear to play a role in extrapulmonary dissemination per se (because the spleens did not differ); rather, this data suggested that urease plays a role in CNS invasion and/or growth in the CNS.

### Role of Cryptococcal Urease in Facilitating C. neoformans *Growth in the Lungs, Spleen, and Brain*

Our next objective was to determine whether urease promoted a direct growth advantage for Cryptococcus in the brain. A single dose of *C. neoformans* was inoculated via intratracheal, intravenous, or intracranial injections and the organ loads were measured on days 1 and 4 after inoculation. These latter experiments now also used *ure1+URE1−1*, the rescued *ure1 C. neoformans* strain in which the wild-type copy of URE1 was stably, ectopically reinserted into the genome. After delivery into the lungs, H99, *ure1*, and *ure1+ URE1−1* all demonstrated similar

pulmonary growth rates during the early phase of infection (Figure 5A). After intravenous injection, the splenic CFU loads of *C. neoformans* strains were identical (Figure 5B). Most importantly, direct intracranial inoculation of H99, *ure1*, and *ure1+URE1−1* demonstrated that urease was not required to grow in the brain (Figure 5C). This latter result is consistent with the previous report that urease is not required for the growth of *C. neoformans* in the brains of corticosteroid-treated rabbits[18] and rules out species-specific differences in our results.

### Role of Cryptococcal Urease in Facilitating Hematogenous Spread and Microcapillary Sequestration

We wished to address the alternate interpretation of Figure 4: urease did facilitate extrapulmonary dissemination as reflected by the brain colonization differences and the early differences in spleen CFU are lost by day 16 (Figure 4). For these experiments, all groups of mice were given an intravenous bolus of *C. neoformans* to uniformly simulate fungemia and analyze the hematogenous spread into the brain. CFUs were analyzed at days 1 and 4. Under these conditions of identical blood fungal loads, nearly 100-fold higher brain CFU was observed with the urease-expressing strains (H99 and *ure1+URE1−1*) compared to *ure1* on both days 1 and 4 after intravenous injection (Figure 6). Interestingly, lung CFU was also 100-fold higher for the urease-producing strains compared to the

**Figure 2.** Photomitograph of *C. neoformans* transfer from blood into the brain 4 weeks after intratracheal infection with 3.87 log CFU of H99 (**a–c**) and 36 hours after intravenous infection with 5.02 log CFU of H99 (**d** and **e**). **a:** Sequestration and proliferation of *C. neoformans* within cerebral MV resulting in discontinuity in capillary wall. **b** and **c:** Progressive invasion of *C. neoformans* (**white arrows**) into the brain parenchyma from MV and the development of the mucoid-cyst (soap bubble); note the contour of necrotic MV and formation of lesion displacing vascular tissues and brain parenchyma. **d:** Growth of *C. neoformans* on the edge of ruptured MV 20 hours after intravenous infection. **e:** Developed microcysts in the brain 36 hours after the intravenous infection. Mucicarmine stain was used to detect cryptococci within the tissues. Original magnifications: ×330 (**a–d**); ×132 (**e**).
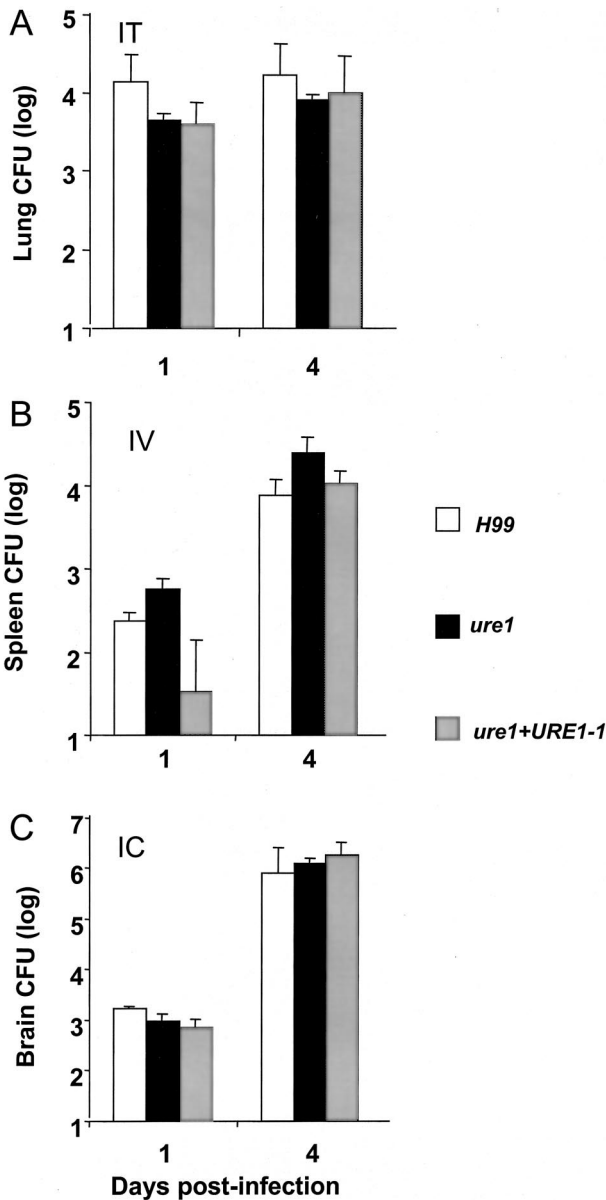
**Figure 5.** Effect of *C. neoformans* urease on organism survival (day 1) and growth (day 4) within organs after direct delivery. Mice were inoculated with 4.05 ± 0.06 log CFU intratracheally (**A**), 3.90 ± 0.05 log CFU intracranially (**B**), or 4.72 ± 0.17 log CFU intravenously of H99 (**C**); *ure1*; or urease revertant *ure1+URE1−1* (*ure1* with reinserted urease gene). CFU in target organs were determined in lungs (**A**), spleens (**B**), and brains (**C**). Bars represent mean ± SE, $n = 5$ to 6 animals per group from three separate matched infections; *, $P < 0.05$.



**Figure 6.** Effect of *C. neoformans* urease on the transfer and subsequent growth of organism in brain and lung after intravenous infection. Mice were injected intravenously with H99, *ure1*, or *ure1+URE1−1* (4.72 ± 0.17 log CFU). Brain and lung CFU were determined at day 1 and 4 days after infection. Bars represent mean ± SE, $n = 4$ to 6 animals per group; *, $P < 0.05$.

urease plays a role in a pathogenesis step that occurs between hematogenous spread and the growth in the brain parenchyma such as microcapillary sequestration and/or *trans*-endothelial invasion.

## Role of Urease Expression in Microcapillary Sequestration by C. neoformans

To determine whether increased sequestration within small capillaries was a mechanism by which cryptococcal urease promoted CNS infection during hematogenous spread, CFU organ loads were measured in numerous organs at 3 hours after bolus intravenous inoculation. This is a long enough period of time for microembolization of cryptococci to occur, but short enough that replication will not significantly affect organ CFU levels (*C. neoformans in vitro* doubling time at 37°C is ~2.6 hours). Consistent with the day 1 and 4 time points, *C. neoformans* accumulation in the spleen at 3 hours was not different in the presence or absence of urease expression (Figure 7A). In contrast, CFU in organs with closed capillary beds (brain, lung, heart, kidney) was more than 10-fold lower for urease-negative *ure1* compared to the urease-positive H99 or *ure1+URE1−1*. We next set up experiments to exclude the possibility that the decreased accumulation of *ure1* in major organs was because of increased susceptibility to serum anti-microbial factors, phagocytosis, or all mechanisms of intravascular clearance combined. All three *C. neoformans* strains were cultured in fresh 90% mouse serum at 37°C for 3 hours, incubated with freshly isolated peritoneal macrophages, or analyzed in the blood 3 hours after intravenous injection. Deletion of the urease gene from *C. neoformans* did not affect the ability of the yeast to survive in serum (Figure 7B), change the number of cells ingested by macrophages (data not shown), or affect the numbers of circulating cryptococci throughout this time period (Figure 7C). Thus, cryptococcal urease activity enhances yeast sequestration within small capillaries, such as within the brain, during hematogenous spread rather than

urease-deficient strain (Figure 6). Comparison of the lung CFU data between Figures 5 and 6 may seem contradictory, but the major difference is the route of entry into the organs. Figure 5A (intratracheal) is measuring direct growth in the alveolar space of the parenchymal tissue of the organ. In contrast, Figure 6 is measuring growth after a process of microcapillary implantation (growth within the vessels of analyzed organ and/or within the parenchyma). Most importantly, the dissemination patterns in the brain and spleen after intravenous inoculation (Figures 5 and 6) are consistent with that observed on day 16 intratracheal-infected mice; supporting the model that
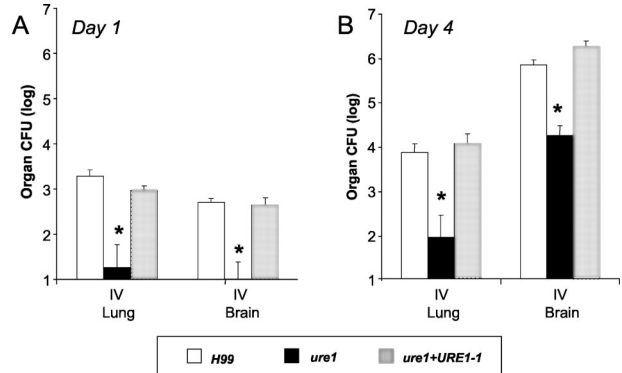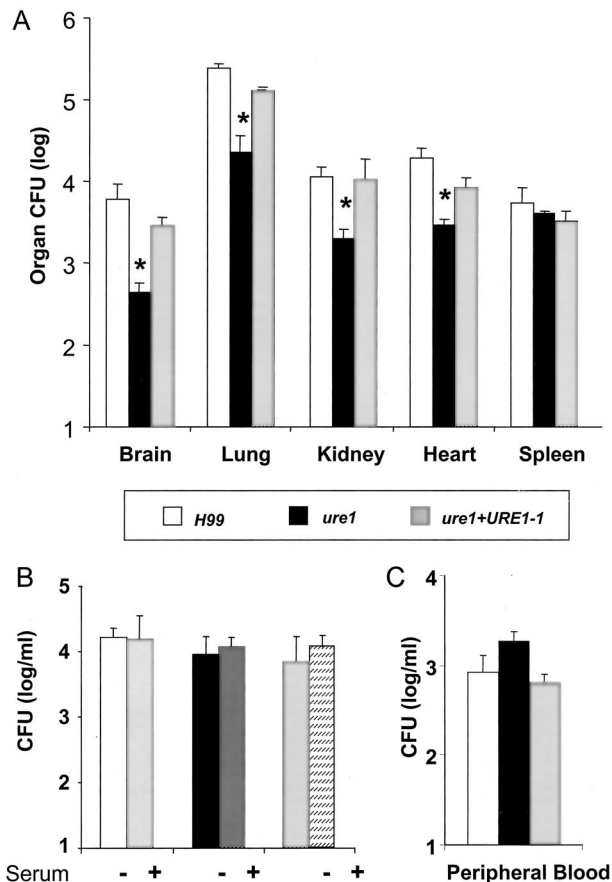
**Figure 7.** Effect of *C. neoformans* urease on vascular sequestration at different organs and numbers of circulating cryptocci after intravenous infection. **A:** Mice were injected intravenously with 5.67 ± 0.09 log CFU of H99, *ure1*, or *ure1 +URE1−*1. There was no significant difference between the inoculi of the various groups. CFUs were determined at 3 hours after infection. *n* = 5 animals per group. **B:** H99, *ure1*, or *ure1 +URE1−*1 were incubated in fresh 90% murine serum for 3 hours in triplicates on 96-well plates on a shaker at 37°C. **C:** Cryptococcal blood CFU at 3 hours after infection (log/ml). *n* = 4 animals/group. Bars represent mean ± SE; *, *P* < 0.05.

providing protection against anti-cryptococcal factors present in the blood.

## Discussion

This is the first study to demonstrate that pathogen-expressed urease can play a role in CNS invasion. In this model of *C. neoformans* infection, urease is critically important for CNS invasion leading to enhanced mortality of infected immunocompetent C57BL/6×129F2 mice. Even though the ability to invade CNS is likely to be mediated by a whole group of cryptococcal virulence factors, our finding was quite unexpected. Urease is a major site-specific virulence factor in bacteria that infects the stomach mucosa or urinary tract.[34−38] Urease enables these microbes to survive in the presence of low pH. Our study demonstrates that urease plays a completely different role in the pathogenesis of infection with *C. neoformans*. Urease promotes cryptococcal sequestration within the microcapillaries, which seems to be a critical step in dissemination to the brain.

Our data support the model that increased intravascular sequestration is an important mechanism by which urease expression facilitates invasion of the brain by *C. neoformans*. As demonstrated in these studies, a 3-hour time frame is not sufficient to produce any significant changes between the strains with respect to growth/survival in mouse serum, *in vitro* phagocytosis by peritoneal macrophages, or numbers of circulating yeast, but a difference in intravascular organ CFU does occur by 3 hours. This model of microcapillary sequestration preceding brain invasion is consistent with other studies using a rat *C. neoformans* fungemia model.[39] In these studies, intravascular granulomas began to form in different organs within hours after intravenous infection.[39] This is also consistent with our observation that urease promotes *C. neoformans* accumulation in organs with closed capillary networks (Figure 7) but not in an organ with an open vascular system, the spleen. In the CNS, cryptococcal invasion appears to occur within brain capillaries in which cryptococci form microemboli, proliferate, and gain access to the brain parenchyma, seemingly via disruption of the vessel wall (Figure 2). This observation in animal models is consistent with the autopsy reports of cryptococcal encephalitis patients that describe *C. neoformans* organisms to be localized in the proximity of cerebral microvessels.[1] Altogether, these data suggest that urease-mediated sequestration of *C. neoformans* to the endothelial surface of microcapillaries of different organs is an important step in pathogenesis of cryptococcal dissemination.

Urease appears to be more critical for the sequestration of *C. neoformans* in microcapillaries the fewer organisms are present in the blood. The requirement for urease in embolization decreases as the level of *C. neoformans* fungemia increases. For example, the most significant role for cryptococcal urease in increasing brain CFU (>1000-fold increase) was observed after intratracheal infection with 4 log CFU (Figures 3a and 4a). During the bolus intravenous injection of 5 log CFU, the effect of urease was less pronounced but still dramatic (100-fold increase in CNS load of H99 compared to *ure1*, Figure 6). There is an even smaller (but still significant) difference in brain CFU after intravenous injection of 6 log CFU (Figure 7). Thus, urease is critical for virulence in infections in which slow pulmonary growth of *C. neoformans* is followed by gradually occurring dissemination and is less important when high bolus doses are injected intravenously during which microembolization may be promoted by increased aggregation of injected yeast cells.

The mechanism by which urease facilitates microvascular sequestration remains unknown. One of the possibilities could be production of ammonia from the urea and/or other nitrogenous products present in host plasma. Yeast-derived urease is extremely efficient in hydrolyzing urea even in significantly diluted normal serum samples.[40] We could further speculate that the production of ammonia in direct proximity of endothelial cells could result in the increased cryptococcal adherence to endothelium[17] either by strictly mechanical effects (change in shape of microvessel), or by an induction of adhesion molecules on the surface of endothelial cells.

Additionally, ammonia could potentially promote endo-thelial cell toxicity and/or affect astrocytes leading to the opening of endothelial cell junctions and weakening the integrity of the blood brain barrier.[7,41] These latter effects however would occur downstream from sequestration and would further increase cryptococcal transfer from the intravascular compartment. Another possibility involves urease-mediated utilization of other nitrogenous moieties found in serum/on the surface of endothelial cells, which in turn would affect the adherence of *C. neoformans* to endothelial cells.

Even though urease has been demonstrated to be critical for the transfer of *C. neoformans* from blood into the brain, urease expression has no role in the survival/ growth of *C. neoformans* in the brain (Figure 4). Only laccase (an other extracellular enzymatic factor pro-duced by *C. neoformans*) has been clearly demonstrated to facilitate intracerebral growth of this organism.[8,42] Con-sistent with the requirement for laccase in CNS infection, our laboratory has performed experiments demonstrating that a laccase-deficient strain produces a minimal CNS infection after intratracheal infection.[43] All *C. neoformans* strains used in this study (H99, *ure1*, *ure1+URE1−*1) were laccase-positive. The ability of these strains to pro-duce laccase was consistent with their ability to grow in the brain on direct injection into the brain. These data indicate that urease and laccase play a synergistic role in CNS invasion, with urease promoting the early steps of *C. neoformans* invasion through brain microcapillaries and laccase promoting yeast survival and replication within the brain parenchyma. Blood-brain invasion and growth of *C. neoformans* in the CNS is a multistep process me-diated by distinct virulence factors at each step, includ-ing at least two extracellular enzymes urease and lac-case.

Even though the effect of urease deletion was striking in *C. neoformans* H99 infection, urease may play different roles in different types of cryptococcal infections. *C. neo-formans* H99 infection is characterized by very rapid dis-semination into the CNS and a paucity of inflammation in the infected areas of the brain. The histological appear-ance of brain cryptococcomas in our model closely re-sembles those found in the subsets of human pathologies of human immunodeficiency virus-positive patients that succumbed to *C. neoformans* infections.[1,15] It also resem-bles the lesions that develop in mice infected with an-other clinical *C. neoformans* isolate.[15] A common element in all of these studies is minimal or completely absent inflammatory response within the CNS despite the pres-ence of infiltrative/invasive cryptococcal growth within the brain parenchyma. In some cases of human CNS inva-sion, referred to as the parenchymal form,[3] mucoid cysts in the brain are found in the absence of meningitis, as it occurs in our model. Our study demonstrates that *C. neoformans* H99 appears on both sides of the blood brain barrier in the absence of an inflammatory reaction or intracellular forms of *C. neoformans*, reported in other studies.[15,17] One of these reports proposed that translo-cation of *C. neoformans* from the blood into the brain occurs via movement of *Cryptococcus*-containing mono-cytes across the endothelial barrier serving as Trojan

horses.[15] In our study, massive translocation of *C. neo-formans* from the blood into brain occurs via microcapil-lary sequestration, extracellular growth, and most likely endothelial disruption without involvement of macro-phages. Thus disseminated *C. neoformans* infection and transfer across the blood brain barrier can occur without meningitis, infection of cerebrospinal fluid, and the in-volvement of intravascular macrophages.

Our final comment would be a speculation on a pos-sibility of the global importance of urease in disseminat-ing infections. High urease expression is an uncommon property of *C. neoformans* compared with other patho-genic yeasts[19] and so is its high CNS tropism. Among other pathogenic yeasts, *Coccidioides immitis* also ex-presses urease and it also causes disseminated disease with CNS infection after spore inhalation.[44−48] Even though *C. neoformans* is the first microbial species re-ported to use urease to facilitate its dissemination into the CNS via sequestration within the microvessels, the pos-sibility exists that urease could be used by other micro-bial species to facilitate their spread into the CNS.

## Acknowledgments

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