Capsule Structure Changes Associated with *Cryptococcus neoformans* Crossing of the Blood-Brain Barrier

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***Cryptococcus neoformans* is a yeast responsible for disseminated meningoencephalitis in patients with cellular immune defects. The major virulence factor is the polysaccharide capsule. We took advantage of a relevant murine model of disseminated meningoencephalitis to study the early events associated with blood-brain barrier (BBB) crossing. Mice were sacrificed at 1, 6, 24, and 48 hours post-intravenous inoculation, and classical histology, electron microscopy, and double immunofluorescence were used to study tissues and yeasts. Crossing of the BBB occurred early after inoculation, did not involve the choroid plexus but instead occurred at the level of the cortical capillaries, and caused early and severe damage to the structure of the microvessels. Seeding of the leptomeninges was not the primary event but occurred secondary to leakage of cortical pseudocysts. Organ invasion was associated with changes in cryptococcal capsule structure and cell size, which differed in terms of magnitude and kinetics, depending on both the organs involved, and potentially, on the bed structure of the local capillary. The rapid changes in capsule structure could contribute to inability of the host immune response to control cryptococcal infection in extrapulmonary spaces. (Am J Pathol 2005, 166:421–432)**

Cryptococcus neoformans is the etiological agent of cryptococcosis, an opportunistic infection that occurs in

individuals with late-stage human immunodeficiency virus (HIV) infection and other cellular immune defects.¹ Even though the morbidity and mortality in these populations has decreased in Western countries,^{2–4} up to 30% of HIV-infected people are still experiencing cryptococcosis in countries of Africa and Southeast Asia where highly active antiretroviral therapy and antifungal agents are not easily available.

While the pathogenesis of cryptococcosis is still not fully understood, there is considerable evidence suggesting the occurrence of a dormant phase of the infection after acquisition of the microorganism via the respiratory route.^{5–7} In immunocompetent hosts the infection is often limited to the lungs whereas in immunodeficient hosts a reactivation may occur that leads to meningoencephalitis and dissemination. Fungemia is a bad prognosis factor during cryptococcosis in both HIV-infected and -non-infected patients^{8,9} and is almost certainly a requirement for fungal dissemination and crossing of the blood-brain barrier (BBB).^{10,11}

Very little is known about the sequential events leading to disseminated meningoencephalitis, the major clinical presentation and cause of death during cryptococcosis. For a long time it was generally believed that invasion of the central nervous system (CNS) followed seeding of the leptomeninges and growth of “microcysts” along the perivascular Virchow-Robin spaces. However, recent work from Olszewski et al¹² emphasized the role of microvascular sequestration in central nervous system invasion but many aspects of the pathogenesis of cryptococcal meningoencephalitis remain unknown.

There is widespread consensus in the field that contact between the polysaccharide capsule of *C. neoformans* and host cells plays a critical role in the pathogenesis of cryptococcal meningitis but the precise role of the capsule in this phenomenon is not well understood. It is well

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demonstrated now that the lack of a capsule^{13–16} and modification of the capsule structure alter the virulence of the strain.¹⁷

In this study, our objective was to investigate the early events associated with *C. neoformans* crossing of the BBB. In particular, we were interested in knowing whether phenotypic changes would be associated with tissue invasion in a relevant model of murine disseminated cryptococcal meningoencephalitis.^{10,18}

Materials and Methods

Animals

Outbred OF1 male mice aged 5 to 7 weeks (Ico: OF1 (IOPS Caw); mean body weight 20 to 30 g, Charles River, Les oncles, France) were used. This strain of mice was selected for its individual susceptibility to *C. neoformans* infection in previous studies showing the clinical relevance of this murine model.^{10,18} Mice were housed 7 to 8 per cage in our animal facilities and received food and water *ad libitum*. This study was conducted in accordance with the EC guidelines for animal care [Journal Officiel des Communautés Européennes, L358, December 18, 1986].

C. neoformans

Strain H99 was grown from frozen stock in yeast nitrogen base broth supplemented with 2% glucose (YNB, Difco Laboratories, Detroit, MI) for 18 hours on a rotary shaker (150 rpm) at 30°C. Yeast cells were always washed three times in sterile phosphate-buffered saline (PBS, pH 7.4) before use. For some experiments, yeasts were labeled with fluorescein isothiocyanate (FITC) as previously described.¹⁹ Briefly, yeast cells were suspended at 2×10^8 /ml in fluorescein isothiocyanate (FITC, Sigma Chemical Co., St. Louis, MO) at 500 μ g/ml in PBS, incubated for 30 minutes at 37°C, then extensively washed in PBS. Cell concentration was adjusted to the desired concentration. In some experiments, yeasts were heat-killed by incubation 1 hour at 80°C before FITC-labeling.

Experimental Infection

The experiments were designed either to study fungal burden in tissue homogenates or to analyze yeast cell structure and localization in fixed tissues. Infection was performed by injection of 100 μ l of the chosen inoculum into the lateral tail vein. Animals were euthanized 5 minutes to 48 hours after inoculation of yeast cells (three mice each time) by injection of pentobarbital (Sanofi Santé animale, Libourne, France).

Measurement of Tissue Burden

For experiments designed to measure tissue fungal burden, a blood sample (0.5 to 1 ml) was withdrawn by cardiac puncture before removal of the organs to allow

culture of buffy coat.¹¹ To avoid contamination of tissues by circulating yeasts, perfusion was then performed by injecting sterile saline (~50 ml) into the left ventricle until whitening of the organs, the right atrium being cut open to allow drainage during the procedure. This procedure did not affect fungal distribution (data not shown). The brain, lungs and spleen, and in some experiments the cardiac and skeletal muscle, kidney, and liver were then aseptically removed, weighed, homogenized in 1 ml of sterile distilled water. Appropriate dilutions of the homogenates were plated (100 μ l) in duplicate onto Sabouraud-chloramphenicol agar Petri dishes and colony forming units (CFU) were enumerated after 48 hours of growth at 30°C. Results were expressed as CFU per gram of organ or per milliliter of blood. The kinetics of blood and tissue burden was then analyzed in three mice at each time interval in at least two independent experiments.

Histological Analysis of Tissue Lesions and Yeast Sizes

In the experiments designed for the histological analysis, mice were transcardially perfused with 20 ml of PBS followed by 20 ml of paraformaldehyde solution (4%). Specimens were then fixed in 4% paraformaldehyde solution, paraffin-embedded, sectioned coronally in 5- μ m thickness and stained with hematoxylin-eosin, Alcian blue, methenamine silver and reticulin. Other specimens were cryopreserved in 40% sucrose, cryosectioned in 7- μ m sections and used for immunofluorescence studies. Each experiment was performed at least twice. For each condition analyzed, at least three coronal sections of each brain were examined, one through the anterior part (olfactory tract), one through the basal ganglia and one through the brain stem and cerebellum. The number of additional sections examined varied depending on the method used and on the frequency of the events studied. The results from independent experiments (two to three mice injected on different days) were pooled unless otherwise stated. The mean size of yeast cells (including the capsule size) was determined using Zeiss Axiovision software (Carl Zeiss Inc., Oberkochen, Germany) by measuring the two diameters of 30 yeast cells seen on tissue sections from two mice infected during independent experiments. Of note, all sections were simultaneously stained to avoid differences in staining intensity.

Electron Microscopy

Cortex, white matter, choroid plexus and leptomeninges were collected, cut in small pieces, fixed in 2.5% glutaraldehyde 0.1 mol/L phosphate (Sorensen's buffer, pH 7.4) and post-fixed in 2% osmium tetroxide solution. After dehydration and embedding in Epon, semi-thin sections were cut and stained with toluidine blue. Ultra-thin sections were stained with uranyl acetate and lead citrate and then examined with an electron microscope (Jeol 100 CX II).

Evaluation of Blood-Brain Barrier Leakage

Mice were infected as previously described. Five minutes after the pentobarbital injection, animals were injected transcardially with a solution of type VI horseradish peroxidase (HRP) (Sigma) (30 mg/ml dissolved in Evans blue 2% in 0.9% saline to provide 100 mg/kg) as previously described.²⁰ The brain was removed less than 1 minute later and fixed by immersion in glutaraldehyde (2.5% in PBS). Coronal sections were then incubated with 3–3' diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA) and hydrogen peroxide according to the manufacturer's instructions. For each study time as well as for the uninfected control mouse, five sections were studied.

Localization of Yeasts with Regard to the Brain Microvasculature

Localization of yeasts with regard to endothelial cells was done using a fluorescein isothiocyanate (FITC)-labeled monoclonal IgG1 antibody specific for cryptococcal polysaccharide (E1,²¹), and an anti-collagen IV antibody (Chemicon, Temecula, CA) that delineated brain capillaries by staining the basal lamina. After microwave treatment and incubation with trypsin-EDTA (Gibco, 37°C, 10 minutes), sections were incubated sequentially with avidin-biotin and protein blocking buffer (Vector), anti-collagen IV antibody (1:200, 1 hour, 37°C), biotinylated anti-rabbit immunoglobulins (Dako, 30 minutes, 37°C), tetramethyl rhodamine isothiocyanate (TRITC)-conjugated streptavidin (Jackson Laboratory, Bar Harbor, MA; 30 minutes, 37°C), and finally FITC-labeled E1 (1 hour, 37°C) after blocking using rabbit immunoglobulins. Each step was followed by 3 washes in PBS. After the last rinse, the coverslips were mounted in Vectashield DAPI (Vector). Negative controls included incubation without the primary antibody and with an irrelevant immunoglobulin at the same concentration. Images were captured on a Zeiss Axiophot microscope (Carl Zeiss) with an Orca ER digital camera (Hamamtsu Photonics, Japan) using Simple PCI (C-Imaging, Compix Inc.) software. For each study time, nine sections from one brain were examined.

Analysis of the Capsule Structure Changes

To analyze the yeast capsular structure over time, we used two monoclonal antibodies that bind different epitopes of the capsular polysaccharide. E1 is an IgG1 murine monoclonal antibody that recognizes an epitope mostly present in *C. neoformans* serotype A capsules²² while CRND-8 (kindly provided by Dr. T. Shinoda, Meiji Pharmaceutical University, Tokyo, Japan²³) is a murine monoclonal IgM antibody raised against *C. neoformans* serotype D that does not bind to serotype A. Sequential incubations with CRND-8, tetramethyl rhodamine isothiocyanate (TRITC)-labeled rabbit anti-murine IgM, and FITC-labeled E1 was performed on the same sections. Sections from various tissues obtained from three mice infected with 10^7 H99 cells during three independent

experiments were studied 1, 6, and 24 hours after inoculation. Results were expressed as the percentage of yeasts to which E1, CNRD-8, or both antibodies were bound. For each slide, the entire tissue section was observed and all visible yeasts were taken into account. In other experiments, yeast cells grown on YPD for 1, 2, 3, 6, or 9 days were fixed with 2% PFA after washings and were subjected to incubation with the same antibodies. Flow cytometry using a XL cytometer (Beckman-Coulter, Hialeah, FL) was performed to analyze 100,000 cells. Yeasts were gated on, and FITC and phycoerythrine (PE) fluorescence was measured under the respective channels. All analyses and quantification were performed using the System II software from Beckman-Coulter.

Statistical Analysis

One-way analysis of variance with Bonferroni's Multiple Comparison Test was performed using GraphPad Prism Version 3.03 for Windows, GraphPad Software, San Diego, CA.

Results

Kinetics of Dissemination after Intravenous Inoculation

As early as 5 minutes after intravenous inoculation, viable yeasts were found in brain, spleen, and lung homogenates. The fungal load within these organs correlated directly with the size of the inoculum (2×10^4 , 2×10^5 , 2×10^6 , and 10^7) (data not shown). We noted that after rapid localization to the brain, the fungal burden decreased slightly and then stabilized for the next 24 hours. After 24 hours, the fungal burden increased rapidly (Figure 1). Analysis of other tissues revealed that fungal load increased with a higher rate in the brain than in the lungs or the spleen (ratio CFU at 48 hours/CFU at 5 minutes = 10.5 in the brain, 5.8 in the spleen, and 2.5 in the lungs after inoculation of 2×10^4 CFU/mouse) (data not

Figure 1. Changes in fungal load with time after intravenous inoculation of 2×10^4 *C. neoformans* strain H99. Outbred mice were sacrificed at various times after inoculation and the CFU were enumerated in brain homogenates (gray bars) and in the blood (white bars). Results expressed as logCFU/g of organ or ml of blood are the mean $\pm$ SD from 3 to 11 mice for each time point.

Figure 2. Fungal load in various organs after inoculation of 10^7 *C. neoformans* strain H99 cells at 1 hour (white bars) and 24 hours (gray bars). Results at each time are the mean $\pm$ SD from three mice.

shown). For the smallest inoculum size tested, a slow decrease in fungemia was observed and the blood culture became negative after a few hours post-inoculation. Dissemination was present in all of the other tissues studied but the fungal burden varied (Figure 2).

Site of the BBB Crossing by *C. neoformans*

Experiments designed to localize yeasts within tissue sections were performed with a high inoculum size (10^7 CFU/mouse) to increase the probability of seeing yeasts in tissue sections. At 5 minutes after inoculation yeast cells were observed in vessel lumen while the majority of those visualized at 24 hours were in brain parenchyma (Figure 3). Some ($< 30\%$) *C. neoformans* cells were observed in the brain parenchyma after just 6 hours. In most of the cases where yeasts were seen intravascularly, the yeasts filled the lumen of the vessel, sometimes distorting the shape of the vessel. Up to 24 hours after inoculation, the yeasts were observed either as singles or in doublets, and no histological change was observed in the surrounding brain parenchyma. At 48 hours, they formed "microcysts" within the brain parenchyma, which coincided with marked edema without inflammatory cell infiltrates.

To characterize the anatomical sites where *C. neoformans* crossed the BBB, all of the yeasts seen in at least five brain sections at the time of sacrifice were enumerated. The vast majority (96% to 100%) was observed in or next to the cortical capillaries of the brain and cerebellum, with only a few yeasts observed in the white matter at 24 hours. In one section, a small cyst was observed at 48 hours post-inoculation in the leptomeningeal area (Figure 4). No yeast cells were observed in the choroid plexus, a structure that always appeared morphologically normal. In fact, no evidence for disruption of the epithelial layer or the basal lamina on reticulin stained choroid

plexus sections was ever observed. Furthermore, the brain stem remained free of yeast cells and tissue abnormalities in all sections examined. When heat-killed FITC-labeled yeasts were inoculated, yeasts were sometimes spotted within brain capillaries 1 hour post-inoculation, but not at later times and never in the brain parenchyma (data not shown).

State of the BBB Associated with *C. neoformans* Crossing

Horseradish peroxidase (HRP) was used to determine whether yeast cell crossing into the brain parenchyma was associated with functional lesions of the BBB. In the brains of uninfected mice as well as in those of mice sacrificed 5 minutes and 1 hour after inoculation, HRP was confined to the lumen of the cerebral blood vessels (Figure 5) except in the median eminence, an area where the vessels are known to be highly permeable²⁰ (this was used as an internal control for our experiments). In the mouse sacrificed 6 hours after inoculation, only one HRP extravasation site could be visualized among five brain sections studied. At 24 hours post-inoculation, both intact capillaries and vessels exhibiting several areas of leakage were observed in the same tissue sections. Vascular hemorrhage and "microcysts" containing yeasts with or without HRP leakage were also observed (data not shown). To better delineate the blood vessels, immunofluorescence studies using antibodies to collagen IV, the major component of blood vessel basal lamina were performed. At 5 minutes and 1 hour post-inoculation, a continuous basal lamina was always observed, sometimes distorted due to the presence of intraluminal yeasts (Figure 3). In contrast, at 24 and 48 hours post-inoculation, intravascular as well as intraparenchymal yeasts were in the vicinity of twisted and apparently damaged basal lamina which often displayed contracted capillary lumen. Electron microscopy revealed extensive damage to the basal lamina and the endothelial cells at 24 hours post-inoculation, although the structures appeared normal at earlier time points (Figure 6).

Yeast Phenotypic Changes Associated with BBB Crossing

The sizes of individual yeast cells were measured in tissue sections stained with methenamine silver to first evaluate phenotypic changes. Yeast cells in the brains of mice sacrificed 6 hours and 24 hours after inoculation were significantly bigger than those in brain sections studied 1 hour after inoculation ($P < 0.001$, Figure 7).

To determine whether the change in yeast size was associated with changes in the structure of the capsular polysaccharide, we took advantage of the specificity of monoclonal antibodies E1 and CRND-8, which are mainly

Figure 3. Brain sections showing the localization of yeast cells at various time intervals after inoculation of 10^7 *C. neoformans* strain H99 cells. Tissue sections from mice sacrificed 5 minutes, 6 hours, 24 hours, and 48 hours after inoculation were stained with Alcian blue (pH 2.5) (left) and stained in red with anti-collagen IV antibody and TRITC-labeled reagent and in green with anti-capsular polysaccharide antibodies by direct (labeled FITC-E1) or indirect (CRND-8 followed by FITC-labeled anti-mouse IgM) labeling. Nuclei are labeled with DAPI (blue) on immunofluorescence (magnification, $\times 1000$).

Alcian Blue

Collagen IV Yeasts

Figure 4. Cortical section of a mouse brain inoculated 48 hours before with 10^7 *C. neoformans* strain H99 cells (methenamine silver staining). The pseudocyst appears ready to extend into the leptomenigeal area.

reactive with *C. neoformans* serotype A- and serotype D-specific polysaccharides, respectively. This set of antibodies was selected because preliminary experiments

performed on brain sections isolated from mice inoculated with *C. neoformans* revealed that CRND-8 is capable of recognizing intraparenchymal yeasts. *In vitro*, more than 95% of the H99 yeasts grown in YPD were stained with E1 whereas less than 0.5% were recognized by CRND-8. Similar results were observed for samples cultured *in vitro* for an extended period of time (9 days, data not shown).

In brain sections of mice sacrificed 1 hour after inoculation, all yeasts reacted only with E1, as evidenced by an intense green ring-shaped staining (Figure 8). At 6 hours after inoculation, half of the yeasts reacted with both antibodies, whereas at 24 hours after inoculation, 70% of the yeasts reacted only with CRND-8 (Figure 9). In yeasts that were double-stained, E1 binding was in the inner part of the capsule and CRND-8 in the outer part. In some cases, superimposition of the binding led to a yellow appearance. Yeasts that were stained with CRND-8 had large capsules and staining often appeared heterogeneous and/or hazy. After 6 hours post-inoculation, several red spots were observed, suggesting active synthesis of CRND-8-reactive material. These red spots could be seen inside yeasts that had capsules exhibiting a CRND-8-positive outer ring (Figures 8 and 10).

Figure 5. Evaluation of the blood-brain barrier leakage by horseradish peroxidase (HRP) extravasation in mice inoculated with 10^7 *C. neoformans* strain H99 cells. Brain section of mice sacrificed 1 hour (**A**), 6 hours (**B**), 24 hours (**C**), and 48 hours (**D**) after inoculation. Extravasation of HRP (**B**, **C**, and **D**) can be seen together with intact capillaries (all panels) (magnification, $\times 400$). Brain tissue sections from control mice or mice sacrificed 5 minutes after inoculation appear as in **A**.

Figure 6. Electron microscopy of brain tissue from mice inoculated intravenously with *C. neoformans* H99. **Left:** One yeast filled the lumen of a cerebral capillary in the brain of a mouse inoculated 5 minutes before sacrifice (magnification, $\times 10,000$). **Right:** Disruption of the vessel wall with neuropil edema around the yeast in the brain of a mouse sacrifice 24 hours post-inoculation (magnification, $\times 4000$).

Changes in Yeast Size and Capsule Structure Associated with Seeding of the Various Tissues

The dramatic changes in size and capsule structure of the yeasts observed in the brain, raised the question of organ specificity. This observation prompted us to measure capsule size and analyze capsule antigenic structure for yeast cells in other organs (lungs, cardiac and skeletal muscles, spleen, liver, and kidneys) at 1 hour, 6

hours, and 24 hours post-inoculation. Compared to *C. neoformans* cells measured in mice sacrificed 1 hour after inoculation, yeasts measured in mice sacrificed 6 and 24 hours after inoculation were significantly bigger in kidney, cardiac, and skeletal muscles ($P < 0.001$, Figure 7). Conversely, only yeasts measured 24 hours after inoculation differed significantly in size from those measured 1 hour and 6 hours after inoculation in spleen, lung, and liver tissue.

Only in the cardiac and skeletal muscles and in the kidney did we observe changes in the capsule structures similar to those observed in the brain over time. Less than 40% of the yeasts reacted with E1 alone at 6 hours and a majority (57% to 86%) reacted only with CRND-8 at 24 hours after inoculation (Figure 9). In the other organs (spleen, liver, and lung), more than 85% of the capsules were still recognized only by E1 at 6 hours. Furthermore, 59% and 83% of the yeasts seen in the spleen and the liver at 24 hours still reacted only with E1, while CRND-8 bound 80% of the yeasts seen in the lungs.

Discussion

In this study we analyzed the early events associated with *C. neoformans* invasion of the brain after intravenous infection of mice and found that crossing of the BBB occurred early after inoculation at the level of the cortical capillaries, did not involve the choroid plexus, and caused severe damage to the structure of the microvessels. Furthermore, dissemination was associated with changes in the structure of the cryptococcal capsule, which varied depending on the tissue seeded. The capsule structure was apparently not influenced by the local fungal load achieved, but rather by the bed structure of local capillaries.

The exact site of central nervous system invasion by *C. neoformans* and the dynamics of the dissemination were determined in a murine model of disseminated cryptococcosis that has been demonstrated to be clinically relevant.^{10,18} In contrast with what is seen during bacterial meningitis,²⁴ crossing of the BBB took place in the cortical capillaries and never in the choroid plexus, confirming recent data obtained by two groups.^{12,25} However, our results also reveal that even though seeding of the leptomeninges may not be the primary lesion in brain dissemination or even a major event leading to cryptococcal meningitis, it may occur as early as 48 hours after inoculation by breakage of cortical "microcysts". In the absence of structural differences between human and mouse brains with regards to choroids plexus structure, these results suggest that similar events may occur in humans. Clinical signs of brain involvement (abnormal mental status, palsies, epilepsy) that could be attributed to encephalitis and are recognized as signs of poor prognosis, are only observed at a rate of approximately 50% (French Cryptococcosis Study Group, unpublished data from the CryptoAD study). Encephalitis may even remain undiagnosed until death because of poor inflammation surrounding the lesions.^{10,26} Thus, meningoencephalitis

Figure 7. Comparison of the yeast sizes in tissue section according to the time of sacrifice. Tissue sections from two mice inoculated with 10^7 *C. neoformans* strain H99 cells were subjected to silver staining, and yeast size including the capsule was determined. Each dot represents the value obtained from each of the 30 yeasts measured, the bar represents the median value.

Figure 8. Comparison of the capsule antigenic structure over time as demonstrated by immunofluorescence studies using double-labeling with two monoclonal antibodies specific for different epitopes on the cryptococcal capsule. Brain tissue sections obtained 1, 6, 24 and 48 hours after inoculation with 10^7 *C. neoformans* strain H99 cells (serotype A) were sequentially incubated with CRND-8 (a murine monoclonal IgM antibody that recognizes sugar epitopes mainly found on serotype D cells), a TRITC-labeled anti-mouse IgM and FITC-labeled E1 (a murine monoclonal IgG1 antibody specific for sugar epitopes mainly found on serotype A cells) (magnification, $\times 1000$).

should probably be the correct term referring to meningeal involvement during human cryptococcosis.

Crossing of the BBB was a very rapid event, as yeasts were seen not only in cortical capillaries, but also within the brain parenchyma as soon as 6 hours after inoculation even though it was a more common observation later on. In the other organs, the tissue structure prevented assessment as to whether the crossing of the endothelium took place as early as 6 hours after inoculation or was delayed compared to the brain. However, we know that dissemination occurred as soon as *C. neoformans* circulated into the blood stream leading to infection of organs with closed and open capillary beds. These results suggest that the capillary structure is not the only determinant for tissue invasion by *C. neoformans*.

Viability was required to cross the BBB since FITC-labeled dead cells were not seen within the brain parenchyma suggesting that active mechanisms are essential. Noverr and colleagues²⁷ recently demonstrated a role for laccase (*CNLAC1*) in promoting escape of *C. neoformans* from the lung, thus favoring extrapulmonary dissemination and brain involvement. In their recent study on the

role of urease, Olszewski and colleagues¹² postulated that urease promoted sequestration and facilitates brain invasion by affecting yeast adherence to endothelial cells, or toxicity toward host cells. They also concluded that translocation of *C. neoformans* from the blood to the brain occurred only via microcapillaries sequestration and endothelial disruption. We showed here that lesions of the BBB and endothelial cell damage existed as early as 6 hours after inoculation by showing HRP leakage, and alteration of the basal lamina and endothelial cells themselves. Therefore, functional and morphological changes of the BBB appear to occur soon after the beginning of cryptococcal fungemia, probably resulting in the breakdown in tight junctions between endothelial cells and leading to brain invasion by intercellular spaces. In a recent study, Chen and colleagues²⁸ demonstrated that physical properties of occludin, a major component of *zonula occludens* were modified *in vitro* after incubation with *C. neoformans*, indicating alteration of tight junctions in brain endothelial cells. However, studies from two groups also suggest that the Trojan horse hypothesis raised by the observation of cryptococci internalized in

Figure 9. Comparison of the changes in cryptococcal capsular antigenic structure in various tissues as demonstrated by the binding of two monoclonal antibodies specific for different epitopes on the cryptococcal capsule. The mean percentage of yeasts ($\pm$ SD) that were stained only with E1 (black bars), only with CRND-8 (white bars) or with both antibodies (striped bars) are recorded. Results were obtained after analysis of tissue sections prepared from three mice infected during three independent experiments.

circulating mononuclear cells during experimental infection,¹⁰ may indeed be valid at least in immunodeficient hosts and with isolates exhibiting specific virulence factors.^{29,30} Finally, a recent study by Chang and collaborators²⁵ showed results indicating that *C. neoformans* cells invade the central nervous system by transcellular crossing of the endothelium of the BBB. Thus, it is likely that several mechanisms participate in brain invasion by *C. neoformans*, but definitive evidence will require further studies.

A major finding of our study is that tissue invasion was associated with rapid changes in yeast size and capsule structures. The initial capsule structure identified as a serotype A specific structure by an E1 binding pattern on all yeasts seen in the brain at 1 hour after inoculation changed to become a ring of E1-positive epitopes surrounded by a ring of CRND-8-positive epitopes at 6 hours (the E1- and CRND-8-specific structures sometimes co-localized in the same yellow ring) and became a CRND-8-specific structure in most of the yeasts observed at 24 hours. Similar observations were made in the kidney, the cardiac and skeletal muscles, and with a delayed kinetics in the lungs, while almost no change in

Figure 10. Double-staining of a yeast observed in the brain of a mouse inoculated 48 hours before with *C. neoformans* H99. The thin green ring corresponds to capsule structures recognized by E1 whereas the red staining corresponds to capsule structures recognized by CRND-8. Note the large granules present inside the yeast cytoplasm suggesting active synthesis of new material.

capsule structure was seen in the liver or the spleen. These results were confirmed in three independent experiments.

The appearance of a new structure around the initial ones seemed in contradiction with the *in vitro* demonstration by Pierini and Doering³¹ who showed that "new capsule formed by mature cells is added at the inner aspects of the existing structure displacing pre-existing material outwards". Several models could explain this apparent discrepancy. A modification in epitope accessibility could be due to changes in capsule density under different growth conditions³² and experimental designs. In addition, the localization of a new structure outside the old one, as seen here, could also be related to the degradation of the initial structure by local factors or by aging. Modifications of the pH through modification in the local CO₂ pressure could affect capsule synthesis^{33,34} and perhaps even capsule structure, although this has only been demonstrated in phagosomes.³⁵ However, it is unlikely that environmental changes are the same in the various organs where capsule structures changes exhibited the same kinetics (brain, cardiac and skeletal muscles, kidney). Aging did not seem to be a factor since rapid capsule structure modifications were observed in muscles and not in the spleen despite similar measurements in fungal burden and multiplication rate. Furthermore, the significant increase in yeast sizes, as well as the visualization of big granules of CRND-8-positive material, inside yeast cells that were surrounded by an inner ring of E1-specific structures and an outer ring of CRND-8-specific structures as soon as 6 hours after inoculation suggested synthesis of new material (and not degradation of the original material even though both events could co-exist). Thus, our results suggest that *C. neoformans* may synthesize polysaccharides *in vivo* to create a new capsule structure by shedding new material from the cell and deposition/association with distal part of the old structure or by new material interspaced with old material, according to two schemes that were discarded by Pierini and Doering³¹ in their *in vitro* demonstration.

In addition to raising the question of how the capsule is synthesized *in vivo* during infection, our results showed that invasion of organs and dissemination were associated with dynamic changes that differ depending on the organ infected. Interestingly, the similarity in the dynamics of capsule structure changes was found among yeasts observed in the organs with closed capillary beds (cardiac and skeletal muscles, kidney, and later, lungs) while yeasts seen in organs with open capillary beds (spleen, liver) appeared almost unchanged in the time frame studied. It should be underlined that changes in the capsule structure have already been observed in the brains of AIDS patients with acute and more chronic meningoencephalitis,¹⁰ thus adding relevance of our observations. The increase in yeast size and the associated modification in capsule structure suggested the existence of an active mechanism that may be triggered during or after the crossing of the endothelium based on the observations made in the brain. *In vitro* experiments with endothelial cells with and without tight junctions may help elucidate the role of host barriers in *C. neoformans* capsule structure changes. Furthermore, it is possible however that prolonging the observation period would have uncovered additional alterations in the capsule structure.³⁶

Microevolutions and phenotypic switching have been reported for *C. neoformans* both *in vitro* and *in vivo*.^{10,37–40} A progressive increase in cell wall thickness as a function of time of infection (2 hours to 28 days) and an heterogeneity in capsule size have been reported during murine pulmonary infection.³⁶ Organ-specific changes have also been evoked.^{40–42} Recently, Garcia-Hermoso and collaborators⁴⁰ showed that the capsule structure evolution differs *in vitro* and *in vivo* and strongly suggest an organ specificity for *C. neoformans* strain selection. Whether serotype A structures such as those recognized by E1 are more adapted to brain invasion remain to be determined. However, it reminded us of the statistically significant difference in the percentage of meningoencephalitis among patients infected with a serotype A isolate (79%) compared to those infected with a serotype D isolate (69%, $P < 0.002$, Mantel-Haenszel test) while there is no significant difference in the percentage of fungemia recorded (66% versus 58%, respectively) (updated data from the epidemiological survey run at the National Reference Center for Mycoses by the French Cryptococcosis Study Group⁴³).

The ability of *C. neoformans* to modify its outer envelope during organ invasion may be an important virulence trait. This strategy is usually adopted by microorganisms to evade the host immune system and to adapt to hostile host environments. Several parasites, viruses and bacteria exhibit antigenic variation during infection.^{44–46} This may allow *C. neoformans* to evade the host defense mechanisms, thus explaining the poor and delayed inflammation usually associated with cryptococcal meningitis^{18,47} and the apparent inefficacy of the spontaneous antibody response that we know is based on a restricted repertoire.^{48,49} In any case, the ability of *C. neoformans* to undergo active and rapid antigenic variability should be kept in mind for the future development of immunotherapy.

The mechanism mediating this antigenic variability is unknown. Using serial analysis of gene expression or SAGE, Steen and collaborators⁵⁰ showed that *C. neoformans* cells present in the cerebral spinal fluid of rabbit with cryptococcal meningitis are actively engaged in protein synthesis, protein degradation, stress response, small molecule transport and signaling but none of the genes recently described involved in the capsule biosynthesis pathway^{51–53} has been identified thus far. It may be that none are triggered in this early machinery and/or that the pathogenesis of cryptococcal meningitis in the rabbit differs from that of disseminated cryptococcosis in the mouse or in humans (one major difference is that rabbits have higher body temperature than both mice or humans). One of the molecular mechanisms evoked or demonstrated for antigenic variability in several pathogens is subtelomeric rearrangements. Several gene families expressing major variant cell-surface proteins are indeed subtelomeric in major human pathogens including the fungus *Pneumocystis carinii* (*P. jiroveci*).^{54–56} However, it is unlikely that such mechanisms would apply since the phenotypic changes are not stable in *C. neoformans* nor are mitotically heritable.⁵⁷

Major advances in the understanding of cryptococcosis pathogenesis have recently been achieved. The virulence of this pathogen is clearly seen as the results of the host-pathogen interaction and not only as the pathogen's attributes.⁵⁸ It was shown that the inability of infected patients to eradicate the yeasts partly resulted from deleterious effects of the capsular polysaccharide.⁵⁹ Evidence is now provided that *C. neoformans* undergoes rapid changes in the structure of its capsular polysaccharide, which could contribute to the inability of the host immune response to control the infection.

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