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Evidence of a Role for Monocytes in Dissemination and Brain Invasion by *Cryptococcus neoformans*▽†

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The pathogenesis of cryptococcosis, including the events leading to the production of meningoencephalitis, is still largely unknown. Evidence of a transcellular passage of *Cryptococcus neoformans* across the blood-brain barrier (BBB) and subsequent BBB disruption exists, but the paracellular passage of free yeasts and the role of monocytes in yeast dissemination and brain invasion (Trojan horse method) remain uncertain. We used our model of disseminated cryptococcosis, in which crossing of the BBB starts 6 h after intravenous inoculation, to study paracellular passage of the BBB. We prepared bone marrow-derived monocytes (BMDM) infected in vitro with *C. neoformans* (BMDM yeasts) and free yeasts and measured fungal loads in tissues. (i) Spleen and lung CFU were >2-fold higher in mice treated with BMDM yeasts than in those treated with free yeasts for 1 and 24 h ($P < 0.05$), while brain CFU were increased (3.9 times) only at 24 h ($P < 0.05$). (ii) By comparing the kinetics of brain invasion in naïve mice and in mice with preestablished cryptococcosis, we found that CFU were lower in the latter case, except at 6 h, when CFU from mice inoculated with BMDM yeasts were comparable to those measured in naïve mice and 2.5-fold higher than those in mice with preestablished cryptococcosis who were inoculated with free yeasts. (iii) Late phagocyte depletion obtained by clodronate injection reduced disease severity and lowered the fungal burden by 40% in all organs studied. These results provide evidence for Trojan horse crossing of the BBB by *C. neoformans*, together with mechanisms involving free yeasts, and overall for a role of phagocytes in fungal dissemination.

Cryptococcus neoformans is an encapsulated yeast responsible for cryptococcosis. It is pathogenic for patients with defective immunity, especially those with AIDS (4). The main clinical presentation is disseminated meningoencephalitis. Cryptococcal meningoencephalitis is always fatal without antifungal therapy, and the death rate is still almost 20% despite adequate antifungal and antiretroviral therapy (33). Brain lesions consist of early endothelial capillary damage followed by fungal proliferation, first in the perivascular spaces and then in the neuropile, with secondary seeding of the meningeal spaces (6). Local inflammation is limited (26, 30), and lesions are described as dilation of the perivascular spaces and as cerebral masses (7, 10, 26).

The pathogenesis of cryptococcosis is still largely unknown (17), while the events leading to the constitution of bacterial meningitis are better understood (25). Bacteremia is critical for the constitution of pneumococcal meningitis (3) and other bacterial meningitides. During cryptococcosis, fungemia is detected in about 50% of human immunodeficiency virus (HIV)-infected patients (15). The correlation between fungemia and dissemination, including brain invasion, has been made for experimental models of cryptococcosis (32), and fungemia is identified as an independent parameter of early mycological failure in humans (15). Brain invasion by *C. neoformans* requires viable yeasts and is thought to occur through the blood-brain barrier (BBB) at the cortical capillary level, not through the choroid plexus (6) as observed for bacterial pathogens (42). The BBB is an anatomical and physiological barrier composed of endothelial cells and pericytes. Endothelial cells are closely associated by numerous tight junctions limiting the circulation between the blood and brain compartments. Monocytes can physiologically cross this barrier through a well-described sequence of tethering, rolling, stopping, and diapedesis. The last step requires a temporary loosening of the tight junctions, involving (i) the junctional adhesion molecules (12), (ii) homophilic interactions of endothelial and leukocyte platelet endothelial cell adhesion molecule 1 (41), and (iii) a temporary destruction of the tight junction protein called occludin (48).

Previous work by a few groups, using in vivo and in vitro models, have suggested three possible routes of BBB crossing by *C. neoformans*, including transcellular passage through endothelial cells, with identification of a specific ligand-receptor interaction (5, 9, 21); a paracellular route between the endothelial cells after mechanical or biochemical disruption of the BBB (6, 9, 46); and finally, a "Trojan horse" method in which cells cross inside a host monocyte.

Several lines of evidence support the existence of the Trojan horse model of BBB crossing. First, *C. neoformans* is a facultative intracellular pathogen and has been shown to survive and multiply inside phagocytes in vitro (53). Second, *C. neo-*

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formans can escape alive from phagocytic cells by an active mechanism of phagosomal extrusion and then invade other phagocytes (2, 36). Third, HIV-infected patients have monocyte dysfunction resulting in reduced anticryptococcal activity (19, 38) and present with a much higher rate of fungemia and meningoencephalitis than that for HIV-negative patients (15). Finally, in the murine model of cryptococcosis, yeasts were seen inside what looked like phagocytes on the outer side of a meningeal capillary, suggesting that *C. neoformans* could have been transported inside circulating phagocytes (10).

Our objective was therefore to demonstrate the existence of a Trojan horse crossing of the BBB by *C. neoformans* and to evaluate the role of monocytes in fungal dissemination.

MATERIALS AND METHODS

Animals. Outbred OF1 male mice aged 5 to 7 weeks (Charles River, l'Arbresle, France) were used for all experiments, except in specific cases involving BALB/c male mice (Janvier, Le Genest-St.-Isle, France). Mice were housed at seven or eight 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 (18a), and experimental protocols were approved by the local committee.

***C. neoformans* strains.** All experiments were done with *C. neoformans* var. *grubii* strain H99, except for coinfection experiments, where KN99α and KN99α NAT were used (44, 45). KN99α and KN99α NAT are congenic strains differing only by the nourseothricin transgene (NAT), which conveys resistance to nourseothricin without modifying the dissemination/virulence properties of the strain (44). This allows specific detection of KN99α NAT colonies in a mix of KN99α and KN99α NAT yeasts by specific growth of the latter on a nourseothricin-containing medium.

Cultures stored at −80°C in 40% glycerol were subcultured on Sabouraud agar and then grown in yeast nitrogen base broth supplemented with 2% glucose (Difco Laboratories, Detroit, MI) for 24 h on a rotary shaker (150 rpm) at 30°C. Yeast cells were harvested, washed three times in sterile phosphate-buffered saline (PBS, pH 7.4), and resuspended at the appropriate concentration in PBS.

BMDM preparation. Bone marrow-derived monocytes (BMDM) were obtained as previously described by maturation of bone marrow cells in the presence of CSF-1, a growth factor secreted by the L929 cell line (18). Briefly, bone marrow cells were flushed from the central femur and tibia cavities of OF1 mice by use of PBS. Cells were seeded at 2×10^6 cells/ml in RPMI 1640 plus GlutaMAX-I medium (Gibco, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum, penicillin-streptomycin (Gibco), and CSF-1 in 75-cm^2 tissue culture flasks (Techno Plastic Products, Trasadingen, Switzerland) at 37°C and 5% CO_2. Nonadherent cells were then harvested after 6 days of culture.

In vitro phagocytosis. In order to study the role of monocytes in *C. neoformans* dissemination, we optimized the in vitro phagocytosis method. Experiments were performed in 96-well flat-bottomed tissue culture plates (Techno Plastic Products) at 37°C and 5% CO_2. The anti-glucuronoxylomannan immunoglobulin G1 (IgG1) monoclonal antibody E1 was used as a source of opsonin (stock solution at 1.5 mg/ml in PBS) (16). BMDM were added at 10^6 cells/well. In preliminary experiments, various ratios of BMDM to yeasts (1:10 to 10:1), various E1 concentrations (1.5 to 6 μg/10^6 yeasts), and various durations of incubation (1 to 3 h) were assessed. As controls, wells containing yeasts plus E1 alone or BMDM alone were incubated under similar conditions. Wells containing yeasts coincubated with BMDM in the presence of E1 (BMDM yeasts), yeasts incubated under the same conditions but without BMDM (free yeasts), or BMDM alone were harvested on ice for further use.

In order to quantify phagocytosed, attached, and free yeasts, as well as yeasts surviving coincubation with BMDM, various experiments were performed. First, microscopic observation was performed to enumerate ingested/attached yeasts or free yeasts. At least four different fields (≥100 yeasts) were counted with a hemocytometer. Second, flow cytometry analysis was performed. Yeast cells were labeled with fluorescein isothiocyanate (FITC) as described previously (6) prior to incubation with BMDM. At the end of the coincubation, the cell suspensions were incubated with allophycocyanin (APC)-labeled anti-CD11b antibodies to detect the BMDM cells (Beckman Coulter, Fullerton, CA). Cells were analyzed for dual fluorescence with Cellquest software after acquisition of 10,000 events on a FACSCalibur flow cytometer (Becton Dickinson). FITC fluorescence of *C. neoformans* is known to be partially quenched inside the macrophage's acidic phagolysosome (28), allowing the assessment of the positions of FITC-labeled yeasts with regard to BMDM according to their level of fluorescence. Thus, APC^+ $FITC^-$ cells were considered free BMDM and APC^- $FITC^{high}$ cells were considered free yeasts. $FITC^{low}$ cells were characterized as phagocytosed yeasts due to partial FITC quenching inside the phagolysosomes. $FITC^{high/intermediate}$ APC^+ cells were considered yeasts adherent to but not internalized by BMDM. Given the quenching associated with internalization, they could also correspond to BMDM containing two yeast cells.

In other experiments, the survival of KN99α yeasts, free or coincubated with BMDM, was studied. Aliquots of 50 μl from the cell suspension were withdrawn at baseline (T0) or after 1 h (T1), 3 h (T3), 6 h (T6), or 24 h (T24) of incubation. The same volume of fresh medium was added at each sampling time. The aliquot was diluted in sterile water, vortexed, and incubated for 1 h at 4°C to release the intracellular yeasts. Appropriate dilutions were plated in duplicate onto Sabouraud agar petri dishes, and CFU were enumerated after 48 h at 30°C. Results were expressed as ratios of CFU of BMDM-KN99α at T*n* to CFU of KN99α at T0. Two independent wells were analyzed during at least three independent experiments.

Experimental infections. Infections were performed by intravenous (i.v.) injection of 100 μl of an inoculum containing 2×10^4 to 2×10^6 yeasts/mouse (see below and Results). The inoculum size was checked by plating appropriate dilutions onto Sabouraud agar-containing petri dishes. Several kinds of experiments were performed to assess different issues, as follows.

(i) The Trojan horse hypothesis was studied by inoculating mice with BMDM-H99 (one injection) or free H99. Mice inoculated with free H99 received in a separate injection the amount of BMDM provided by the injection of BMDM-H99.

(ii) The coexistence of Trojan horse crossing and crossing as free yeasts was studied by injecting mice with an inoculum composed of BMDM-KN99α and free KN99α NAT at a 1:1 ratio.

(iii) The potential alteration of BBB crossing by a preestablished infection obtained by a high inoculum known to provide parenchymal lesions and BBB disruption at day 2 (d2) postinoculation was studied (6). Two groups of mice were first inoculated at d0 with 2×10^6 KN99α cells and then at d2 with 2×10^4 free KN99α NAT cells (free-d2 group) or with 2×10^4 BMDM-KN99α NAT cells (BMDM-d2 group). Two control groups of naïve mice were inoculated only with 2×10^4 free KN99α NAT cells (free-d0 group) or with 2×10^4 BMDM-KN99α NAT cells (BMDM-d0 group).

(iv) Finally, the role of phagocytes at a later stage of fungal dissemination was studied. Clodronate-containing liposomes were used to induce sustained monocyte depletion after the onset of infection. Clodronate (provided by Roche Diagnostics GmbH, Mannheim, Germany) (54) has been shown to induce nearly complete monocyte elimination 18 h after i.v. injection, with progressive monocyte repopulation starting 24 h after injection and reaching normal levels 3 to 4 days later. Thus, to obtain sustained monocyte depletion, clodronate liposomes (200 μl) were injected daily for four consecutive days, starting 72 h after inoculation with 10^5 H99 yeasts.

In all experiments, animals were euthanized by intraperitoneal injection of pentobarbital (Sanofi Santé Animale, Libourne, France). Since crossing of the BBB is known to start 6 h after inoculation in this model, animals were sacrificed 1, 6, and 24 h after inoculation to study the early events (experiments 1, 2, and 3) and on day 7 (24 h after the last clodronate injection) to study the late events (experiment 4).

To avoid contamination of tissues by circulating yeasts, infusion with 50 ml sterile saline was done through the left ventricle as described previously (6). The brain, lungs, and spleen were then aseptically removed, weighed, and ground in 1 ml of sterile distilled water. Serial dilutions of the homogenates were plated in duplicate onto Sabouraud agar petri dishes. In the KN99α-KN99α NAT coinfections, the total numbers of KN99α and KN99α NAT colonies (Sabouraud agar) and of KN99α NAT colonies (Sabouraud agar plus nourseothricin at 100 μg/ml) were determined. CFU were enumerated after 48 h at 30°C.

All experiments were repeated at least three times, with three mice per group, unless otherwise specified. Results were expressed as the ratios of the mean CFU/g of organ for the experimental group (BMDM yeasts) to that for the control group (free yeasts) unless otherwise stated (results are means ± standard deviations [SD]).

Statistical analysis. Statistical analysis was performed using the STATA 10.0 statistical package (Stata Corporation, College Station, TX). Variables were compared using the nonparametric Wilcoxon signed-rank test or the Kruskal-Wallis test, depending on the number of groups. P values of <0.05 were considered significant.

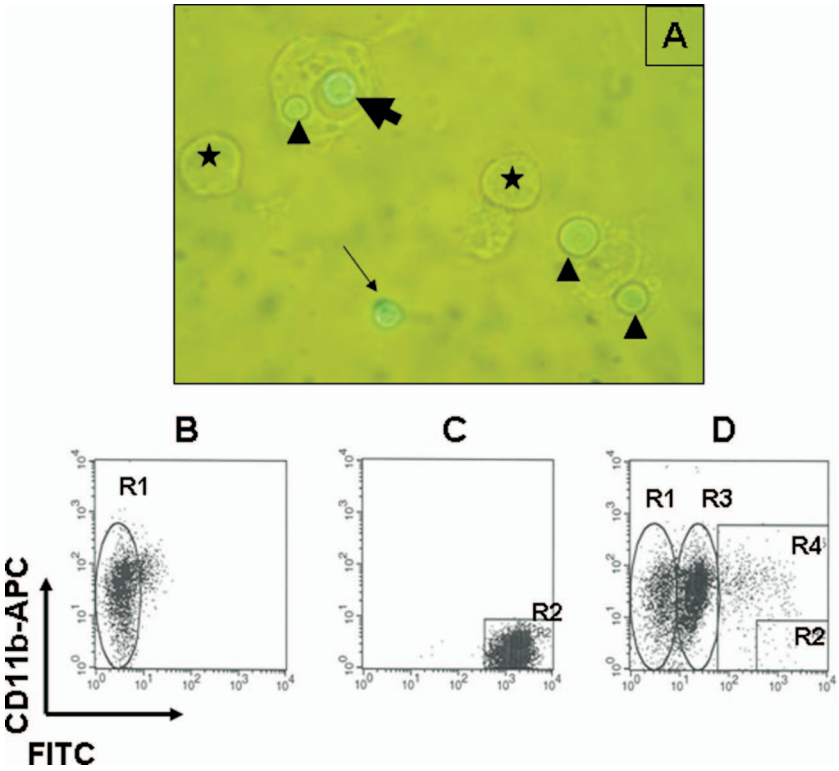

FIG. 1. Representative in vitro phagocytosis of *C. neoformans* by BMDM. BMDM were incubated with FITC-labeled *C. neoformans* strain H99 and monoclonal antibody E1 (1.5 μg/10^6 yeasts) for 90 min at a BMDM/yeast ratio of 3:1. (A) Epifluorescence microscopy. The bright FITC staining is partially quenched after phagocytosis, allowing the distinction between free yeasts (thin arrow), intracellular yeasts (arrowheads), and adherent yeasts (large arrow). Uninfected BMDM (black stars) are also visible. Magnification, ×1,000. (B to D) Flow cytometry analysis of CD11b-APC-labeled BMDM (B), FITC-labeled *C. neoformans* H99 (C), and BMDM-H99 inoculum prepared as described above (D). CD11b-APC-labeled BMDM are seen in gate R1, free FITC-labeled yeasts ($FITC^{high}$ APC^{-} cells) are seen in R2, phagocytosed yeasts ($FITC^{low}$ APC^{+} cells) are seen in R3, and BMDM-adhering yeasts or just internalized yeasts ($FITC^{high/intermediate}$ APC^{+} cells) are seen in R4.

RESULTS

Evidence for the existence of Trojan horse crossing by *C. neoformans*. In preliminary experiments, phagocytosis by BMDM was optimized in order to limit (i) the number of free yeasts, which would hamper the demonstration of the role of the monocytes in *C. neoformans* BBB crossing; and (ii) the number of yeast aggregates and of BMDM containing more than two yeast cells, which would make them less likely to enter small capillaries and then to cross the BBB. The experimental conditions did not allow the preparation of an inoculum of $>10^5$ yeasts/mouse to avoid BMDM clumping (data not shown) or $<2 \times 10^4$/mouse because of the threshold of CFU detection.

Both microscopy and flow cytometry showed that a BMDM/yeast ratio of 3:1 in the presence of E1 at 1.5 μg/10^6 yeasts for 90 min was associated with at least 75% of yeasts being internalized/adherent to BMDM in more than five independent experiments (a representative experiment is shown in Fig. 1). To determine if phagocytosis by BMDM altered fungal multiplication, the viability of KN99α incubated in the presence or absence of BMDM was studied in vitro. CFU were not different in both groups at all times studied (data not shown).

We then studied the Trojan horse hypothesis in vivo. Mice inoculated with BMDM-H99 had higher fungal burdens in the spleen and lungs than did control mice inoculated with the same amount of free H99, whatever the time of sacrifice, and this difference was significant 1 h and 24 h after inoculation (CFU ratio, ≥2.0; $P = 0.0369$). In the brain, fungal loads were similar at 1 h and 6 h in both groups, but they were significantly higher at 24 h for mice inoculated with BMDM-H99 than for those inoculated with free H99 (CFU ratio at 24 h = 3.9; $P = 0.0369$) (Fig. 2; see Table S1 in the supplemental material).

Concomitant BBB crossing of *C. neoformans* as free cells and by the Trojan horse method. We then designed experiments to determine if the "Trojan horse" hypothesis for BBB crossing could occur together with mechanisms involving free yeasts. The fungal burden of animals inoculated with equal numbers of free KN99α and BMDM-KN99α NAT at a final fungal inoculum of 4×10^4 (one experiment) or 1×10^5 (two experiments) was analyzed.

Both KN99α and KN99α NAT were found in the brain 1, 6, and 24 h after inoculation, with a nonsignificant trend toward more KN99α NAT at all times in the three independent experiments (see Fig. S1 in the supplemental material). This was confirmed in three mirror experiments with BMDM-KN99α and free KN99α NAT (data not shown).

Monocytes participate in brain invasion by a secondary strain postinfection. We then compared the courses of brain infection after inoculation with BMDM-associated or free KN99α NAT at d0 in two groups of naïve mice (BMDM-d0 or

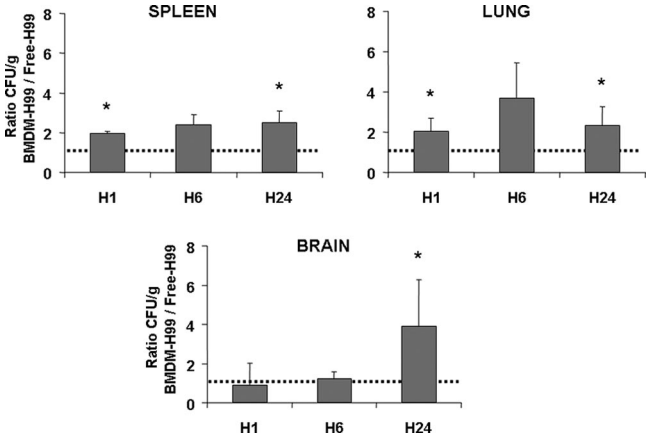

FIG. 2. Effect of inoculation with yeasts internalized in monocytes on *C. neoformans* tissue burden. Mice were inoculated with 2×10^4 BMDM-H99 or 2×10^4 free H99 (free-H99) and sacrificed 1, 6, and 24 h after inoculation. Results are expressed as ratios of the mean CFU/g of organ for the experimental group (BMDM-H99) to that for the control group (free-H99) (data are means ± SD for three mice in each group). The dotted line denotes a ratio of 1. Two independent experiments included the three time points, and the last one included only the 1-h and 24-h study points. *, $P < 0.05$.

Group	Inoculation at day 0 (D0) and day 2 (D2) with		
	KN99α	FreeKN99αNAT	BMDM-KN99αNAT
BMDM-d0	-	-	2 x 10^4 at d0
BMDM-d2	2 x 10^6 at d0	-	2 x 10^4 at d2
Free-d0	-	2 x 10^4 at d0	-
Free-d2	2 x 10^6 at d0	2 x 10^4 at d2	-

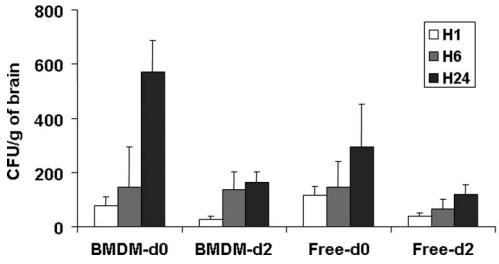

FIG. 3. Effect of established cryptococcal infection on brain invasion by a second isolate of *C. neoformans*. Inoculation with 2×10^4 free KN99α NAT or BMDM-KN99α NAT cells was done on day 0 to naïve mice (free-d0 or BMDM-d0 group) or mice injected 2 days before with 2×10^6 KN99α cells (free-d2 or BMDM-d2 group), as summarized in the table. Mice were sacrificed 1, 6, and 24 h thereafter. Results are expressed as CFU/g of brain (data are means ± SD for three mice). Only CFU data resulting from KN99α NAT inoculation are presented.

free-d0 group) and at d2 in two groups of mice with previously established cryptococcal infection (BMDM-d2 or free-d2 group).

Viable yeasts were enumerated in all groups 1, 6, and 24 h after inoculation (Fig. 3). CFU enumerated in the brains of mice with preestablished infection were significantly lower than those in the brains of naïve mice, and this was true for mice inoculated with free yeasts or BMDM yeasts at 1 h and 24 h postinoculation ($P = 0.0495$). Fungal loads measured in the brains of BMDM-d2 mice were significantly higher than those in free-d2 mice at 6 h postinoculation ($P = 0.0495$). The kinetics over the 24 h differed between the four groups. The increase in CFU recorded at 6 h compared to that at 1 h postinoculation was 5-fold for BMDM-d2 mice, compared to 1.9-fold for BMDM-D0 mice, 1.8-fold for free-d2 mice, and 1.3-fold for free-d0 mice, suggesting differences in yeast multiplication or BBB crossing.

Delayed and sustained phagocyte depletion reduces fungal dissemination. Fungemia can occur after transient clearance of the yeasts, suggesting that yeasts can recirculate freely or be associated with phagocytes (32). The BMDM yeast approach was useless to answer the question of the role of phagocytes at later stages of the infection, whereas phagocyte depletion seemed more appropriate. Sustained phagocyte depletion was performed during an already disseminated cryptococcal infection, and mice were sacrificed on day 7 after inoculation (Fig. 4; see Table S2 in the supplemental material). All control animals were ill, with ruffled hair and numerous macroscopic abscesses in the brain and spleen at autopsy. Mice injected with clodronate were clinically less ill and had fewer or no visible macroscopic abscesses. Spleens from control mice were significantly heavier than those from clodronate-injected mice (169 ± 13 mg versus 126 ± 7 mg; $P = 0.016$). In depleted mice, fungal burdens were reduced >40% in all organs studied, including the brain, compared to those in control mice ($P = 0.039$; pooled results from two independent experiments involving outbred OF1 mice and one experiment using BALB/c mice).

DISCUSSION

As discussed for bacterial or viral pathogens (25, 47), several mutually exclusive or concomitant mechanisms are thought to allow BBB crossing by *C. neoformans*. Transcellular passage through endothelial cells has been demonstrated in vitro (5) and evidenced in vivo during experimental cryptococcosis (10). Paracellular passage requires disruption of the BBB by mechanisms that are mostly unknown during cryptococcosis. *C. neoformans* could secrete proteases that could alter the integrity of the BBB (8, 46, 49). Sequestration of the yeasts in the cortical capillaries could produce mechanical disruption, and enzymes such as urease could participate in this event (46). Furthermore, secretion of vascular endothelial growth factor has been observed during cryptococcosis (11), and it is known to be associated with BBB leakage. Whatever the mechanisms involved, the extensive damage to the endothelial layer and BBB leakage observed in vivo (6) could allow translocation of yeasts, either freely or inside blood monocytes.

The Trojan horse hypothesis has been evoked but never demonstrated until now. Most experimental studies have focused on the role of lung macrophages as a vector for *C. neoformans* dissemination and possibly for BBB crossing in models using virulence-altered yeasts (22, 23, 34, 49). In the clinical setting, however, patent pneumonia is often lacking, which could limit the role of alveolar macrophages, while fungemia is reported for almost 50% of HIV-infected patients (15). HIV infection was recently shown to increase the capacity of peripheral monocytes to cross the BBB into the brain (56), potentially contributing to the larger proportion of meningoencephalitis cases in HIV-positive than in HIV-negative patients (15). In our experimental model, yeasts have been ob-

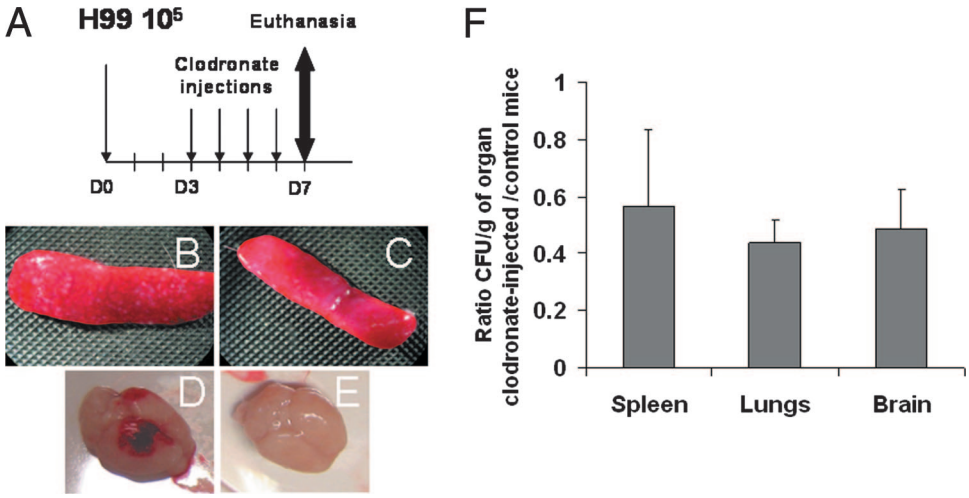

FIG. 4. Effects of delayed and sustained monocyte depletion on the course of cryptococcal infection. (A) Mice were infected with 10^5 H99 yeasts and treated by daily i.v. infection with clodronate liposomes (depleted group) or with PBS (control group) for 4 days. Macroscopic examination of representative organs from mice in the control group showed multiple abscesses in the spleen (B) and hemorrhages in the brain (D), but mice in the depleted group showed no visible lesions in the spleen (C) or the brain (E). (F) Ratios of fungal loads measured in the spleens, lungs, and brains of depleted and control mice (data are means ± SD for three animals per group from three independent experiments).

served in the blood, circulating either inside mononuclear cells or freely (10, 32). The magnitude of fungemia recorded on day 1 postinoculation is correlated with brain, spleen, and lung burdens (32), making the peripheral monocyte a potential "vector" for fungal dissemination and brain invasion. In a study by Kawakami et al., there was no reduction in cerebral infection when mice treated with anti-CD11b antibodies were inoculated i.v. to study the role of phagocytes in the blood in brain passage (22). However, the effect of the anti-CD11b treatment on circulating monocytes or fungemia was not assessed. In another study, H99-infected monocytes induced brain infection in one of three recipient mice after i.v. inoculation, in contrast to three of three mice inoculated with H99-infected alveolar macrophages (49). However, in these experiments, monocytes were 300-fold less infected than the alveolar macrophages. Thus, the role of monocytes in dissemination and BBB crossing was still unclear.

The use of cultured BMDM and the assessment of conditions leading to a reproducible and high level of infection with *C. neoformans* allowed us to demonstrate a role of monocytes in fungal dissemination and BBB crossing by *C. neoformans*. However, our study has some limitations that need to be mentioned. First, the fungal load measured in tissue by CFU enumeration is the result of tissue invasion, yeast multiplication, and the host response. The maximum number of BMDM injected/mouse was under the threshold of histological detection, thus preventing direct observation of yeast transport by monocytes and control of the fact that when they crossed, yeasts were still inside monocytes. The fact that BMDM yeasts tended to multiply less than free yeasts under our in vitro conditions probably ruled out the possibility of better proliferation inside BMDM, as described for other conditions with monocyte-derived human macrophages (14). Second, despite the high level of phagocytosis achieved (>75%), the free yeasts present in the inoculum could have blurred the final "picture." Additionally, *C. neoformans* has been shown in vitro to escape from macrophages, which could have modified the proportion of free/phagocytosed yeasts in the inoculum (2, 36). However, the escaping phenomenon involved <10% of the phagocytes and was more frequent with human macrophages than with murine macrophages and with *Clostridium gattii* than with *C. neoformans* (35). Finally, depending on the inoculum preparation, different fungal and host genes could be triggered, modifying fungal multiplication or survival, as suggested by others (49). Nevertheless, we observed a reproducible threefold increase in the fungal burden measured in the brain 24 h after inoculation with BMDM- associated yeasts compared to the burden after inoculation with free yeasts. This suggests that the Trojan horse hypothesis is valid for *C. neoformans* crossing of the BBB.

Since other means of BBB crossing involving free yeasts have been evoked or demonstrated, we studied their coexistence with Trojan horse crossing by using resistant and susceptible yeasts of equal virulence (44), injected together either as free cells or associated with BMDM. Both BMDM-associated and free yeasts were detected in the brain, with a trend toward a larger number of BMDM-associated ones at all times studied. These experiments, together with other experiments involving various mutants or congenic strains (43, 44), suggest that multiple strains can invade the brain concomitantly. These data support the idea that mixed infections can occur in humans (37) and that *C. neoformans* can use multiple means of crossing.

We then wondered if prior brain infection by *C. neoformans* could alter the subsequent kinetics of BBB crossing by a second strain. Prior infection could modify several parameters that could subsequently interfere with BBB crossing and fungal multiplication, including (i) disruption of the BBB and its tight junctions between endothelial cells and modification of the endothelial surface, which could increase BBB crossing (6); and (ii) alteration of local immunity, with diminished secretion of chemokines by endothelial

cells, as demonstrated in vitro (40). Whatever the underlying mechanisms, prior infection modified the course of brain invasion by a second strain. A smaller number of yeast cells was recovered from the brain, regardless of the inoculum preparation (BMDM-associated or free yeasts), than what was observed with the same inoculum in naïve mice at all time points except the 6-h time point. As demonstrated in naïve mice, inoculation with BMDM yeasts still increased the fungal load in the brain compared to inoculation with free yeasts in the setting of prior infection. Moreover, CFU recovered from the brains of mice secondarily inoculated with BMDM yeasts (BMDM-d2 group) were higher (2.5 times) than those expected from the data obtained for mice inoculated with free yeasts (free-d2 group). These data suggest increased or earlier BBB crossing, since yeast multiplication in the brain has been shown to occur after BBB crossing in this model (6). Overall, our results provide further evidence of the existence of monocyte-associated crossing of the BBB by *C. neoformans*. They suggest, however, that when mixed infections occur in humans, they may be more likely to occur at once than to represent subsequent reinfection. Studies are now required to further dissect the molecular events associated with BBB crossing by *C. neoformans* and to study how the modification of local immunity affects interactions of yeasts with the BBB.

Beyond its involvement in early BBB crossing, the monocyte also appeared to be an early dissemination vector in these experiments. The kinetics of fungal load after inoculation with BMDM-associated yeasts differed in the spleens and lungs from what was observed in the brain. Indeed, more yeasts were recovered in the spleen and lungs as soon as 1 h after inoculation of mice inoculated with BMDM yeasts than from those inoculated with free yeasts, while this was observed only at 24 h in the brain. This discrepancy could be related to functional differences of leukocyte-trafficking targets, between the "immunological sanctuary" with limited cell trafficking (brain) and the "filter" organs with intensive circulation of immune cells (spleen and lungs), rather than to differences in capillary bed structures, since both the lungs and the brain share a continuous bed structure (24). This discrepancy is reminiscent of previous experiments in which fungal loads progressed differently in these three organs (32) and crossing of the various endothelia by yeasts was associated with rapid changes in the capsule structure which varied depending on the organ seeded (6). Our results suggest that monocytes have a role in fungal dissemination and brain invasion but that molecular events could differ depending on the organ invaded.

Cryptococcal infection is associated with limited inflammation, but low concentrations of proinflammatory cytokines and chemokines, such as tumor necrosis factor alpha, gamma interferon, macrophage inflammatory protein 1α, and monocyte chemoattractant protein 1, during cryptococcosis could enhance leukocyte recruitment and subsequent organ reseeding by infected phagocytes (1, 20, 29, 30, 55). In experiments performed in the early 1980s, Monga showed that depletion of tissue macrophages by treatment with silica prior to infection enhanced susceptibility to infection (39). Using different experimental designs and more recent tools, others have demonstrated the role of tissue macrophages in fungal dissemination (23, 34, 49). In our experiments, late phagocyte (blood monocyte plus circulating phagocyte) depletion obtained by clodronate injections (27, 52) consistently reduced clinical manifestations and fungal burdens in all organs studied. Previous data from our group demonstrated that late fungemia can occur even after transient clearance of the yeasts, suggesting the possibility of blood seeding from infected tissues (32). The presence of intracellular yeasts visualized in infected tissues suggests a role for phagocytes in recirculation (10). The diminution of fungal load in all organs studied provides further evidence of this phenomenon. It also suggests that reseeding was equally reduced, regardless of organ function and endothelium structure. Clodronate is known to induce concomitant depletion of tissue macrophages, but no information is available concerning depletion of perivascular macrophages after i.v. injection (27, 52). Overall, the role of phagocytes is complex (13). We show here again the predominant role of phagocytes in reseeding tissues rather than eradicating yeasts (50). This could have interesting implications for vaccine development. Indeed, by driving *C. neoformans* into phagocytes, antibodies to capsular antigens could promote organ dissemination with these "Trojan horses." Knowing that tissue lesions observed during immune reconstitution inflammatory syndrome contain yeasts inside $CD68^+$ cells, one could also raise the question of whether phagocyte-mediated reseeding participates in the severe clinical manifestations related to this syndrome in the setting of HIV infection or solid organ transplantation (31, 51).

Our data provide strong evidence for Trojan horse crossing of the BBB by *C. neoformans*, together with other mechanisms involving free yeasts, and the detrimental effect of phagocytes on the course of cryptococcosis. *C. neoformans* has well-known antiphagocytic, anti-inflammatory, and immunomodulatory properties (13). The monocyte subversion reported here appears to be another effective tool to escape the host defenses.

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