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Experimental Model of Intracerebral Infection with *Cryptococcus neoformans*: Roles of Phagocytes and Opsonization

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A murine model of intracerebral (i.c.) infection with *Cryptococcus neoformans* in which naive mice receiving an i.c. fungal inoculation developed a severe disease has been established. The effect was strictly dependent on the number of microorganisms injected and evolved as lethal meningoencephalitis. Murine susceptibility to i.c. infection with *C. neoformans* was enhanced by treatment with chloroquine and colchicine, agents known to greatly affect the host phagocytic compartment. Furthermore, the life spans of both naive and drug-treated mice were significantly augmented when opsonized fungi were injected. Therefore, phagocyte-mediated mechanisms are likely involved in local resistance to i.c. infection with *C. neoformans*. Further support for this conclusion was supplied by in vitro data showing that microglial cells were proficient anticryptococcal effectors, provided opsonized microorganisms were used.

Cryptococcus neoformans, a yeastlike organism, is a significant cause of opportunistic infections with a marked predilection for the central nervous system (CNS) (20). Cryptococcosis may occur as an asymptomatic pulmonary infection; however, immunocompromised hosts may present a fatal disseminated disease generally manifested as meningitis (33). The disease is frequently observed in patients treated with corticosteroids or those with hematologic malignancies or AIDS. In AIDS, it is the fourth most common life-threatening disease (4, 9).

Numerous in vitro studies have demonstrated that polymorphonuclear leukocytes (PMN), monocytes, and macrophages can ingest encapsulated cryptococci opsonized with either complement or anticryptococcal antibodies (6, 7, 19, 21, 22). While PMN are clearly fungicidal, macrophages are less effective in killing *C. neoformans* in vitro (8, 27), possibly because of the in vitro conditions used to generate activated macrophages (10, 14, 23). In addition, it has been reported that natural killer cells can inhibit the growth of *C. neoformans* in vitro (17, 28, 31). Despite the importance of innate immunity, the capacity of the host to mount an adequate cell-mediated immune response also plays an important role in resistance to cryptococcosis (3, 25, 29). The importance of such an immune response has been particularly evident in murine models (18) and in AIDS patients (4, 9); in these models and patients, helper T lymphocytes are affected.

We recently immortalized in vitro primary microglial cultures with a recombinant retrovirus carrying v-*raf* and v-*myc* oncogenes (1). The continuous cell line BV-2 retains the morphological, phenotypic, and functional properties ascribed to primary microglia (12, 15). Potent anti-*Candida albicans* activity is exhibited by BV-2 cells either in vitro or in vivo following intracerebral (i.c.) transfer into syngeneic mice which, in fact, resist a lethal local injection with *C. albicans* (2). While the local mechanisms of host defense against *C. albicans* are still unclear, recent evidence has been provided that interleukin-1 and a low-virulence strain of *C. albicans*, known to induce a protective antifungal state when given systemically (36), both act as potent immunomodulatory signals within the brain compartment (26).

To gain more insight into the host-parasite relationship occurring at the cerebral level, we established an experimental model of i.c. infection with *C. neoformans* that evolved into lethal meningoencephalitis. We showed that upon opsonization, the in vivo pathogenic burden of the fungus was reduced, while its in vitro susceptibility to microglia-mediated antifungal effects was increased. An impairment of cerebral host defenses against *C. neoformans* was observed in mice exposed to chloroquine plus colchicine, agents known to block the functional properties of phagocytic cells (5, 11, 13).

MATERIALS AND METHODS

Mice. Female CD-1 mice, 6 to 8 weeks of age, were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy.

***C. neoformans*.** An encapsulated strain of *C. neoformans*, ATCC 11240, was used. Stock cultures were maintained by biweekly passages on Sabouraud dextrose agar. The plates were kept at room temperature. Yeast cells were harvested from the agar plates, washed twice in saline by low-speed centrifugation (1,000 × *g*), and diluted to the appropriate concentration in RPMI 1640 medium or saline prior to being used in the in vitro or in vivo assays. *C. neoformans* was opsonized as follows. Microorganisms (2×10^8) were incubated for 20 min at 37°C in 200 μl of fresh mouse serum. The excess serum was removed, and appropriate dilutions were prepared in RPMI 1640 medium or saline for the assays.

Cell line. The BV-2 microglial cell line used in this study was obtained as previously described (1). In brief, primary microglial cultures were infected with a v-*raf* and v-*myc* oncogene-carrying retrovirus and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf

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serum, gentamicin (50 μg/ml), and L-glutamine (2 mM) (complete medium). Biweekly, cells were detached by vigorous shaking and fresh cultures were started at a concentration of 5×10^4 cells per ml.

Drug treatments. Chloroquine (25 mg/kg) and colchicine (1 mg/kg) (Sigma, St. Louis, Mo.) were dissolved in pyrogen-free saline and injected into mice intraperitoneally 5 days before i.c. challenge.

i.c. inoculations. i.c. inoculations were carried out as previously described (34). In brief, yeast cells were suspended in pyrogen-free saline and injected (30 μl per mouse) into the brain, 1 mm laterally and posteriorly to the bregma at a depth of 2 mm, with a 0.1-ml glass microsyringe and a 27-gauge disposable needle. Mice recovered from trauma within 30 to 60 min. Surgical mortality was less than 3% and always occurred within 1 to 5 min after injection.

Phagocytosis assay. Opsonized and unopsonized *C. neoformans* microorganisms (2×10^7 cells per ml) were incubated with BV-2 cells (10^6 cells per ml) for 2 h at 37°C. The excess *C. neoformans* was removed by centrifugation of the cell suspension on a Ficoll cushion at $300 \times g$ for 10 min. The cells at the interface were recovered and washed. *C. neoformans* uptake was directly evaluated in Giemsa-stained cytospin preparations. A minimum of 200 cells were scored, and any cells containing one or more particles were counted as phagocytic.

CFU assay. BV-2 cells were plated at different concentrations (0.1 ml per well) in 96-well plates (Corning Glass Works, Corning, N.Y.) and infected with 0.1 ml of *C. neoformans* (5×10^3 cells per ml). After 8 h of incubation at 37°C under 5% CO_2, Triton X-100 (0.1% final concentration) was added to the wells and the plates were vigorously shaken. Serial dilutions from each well were made in distilled water and plated on Sabouraud dextrose agar. The colonies were counted after 48 h of incubation at room temperature. Control cultures consisting of *C. neoformans* incubated without effector cells contained between 100 and 130% the CFU initially plated. Results were expressed as the percentage of CFU inhibition according to the following formula: percentage of CFU inhibition = 100 − ([CFU in experimental group/CFU in control cultures] × 100).

Quantitation of *C. neoformans* in organs. Organs from each mouse (three mice per group) were removed aseptically and placed in a tissue homogenizer with 3 to 6 ml of sterile distilled water. The number of CFU in the specimens was determined by a plate dilution method with Sabouraud dextrose agar. After 48 to 72 h of incubation at room temperature, the colonies were counted and the results were expressed as the number of CFU per organ.

Histopathological analysis. Mice were sacrificed 15 days after i.c. infection with *C. neoformans*. Brain specimens were fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. Sections (5 μm) were cut and stained with periodic acid-Schiff stain by standard procedures.

Statistical analysis. Differences in survival times were determined by the Mann-Whitney U test. Differences in the percentages of CFU inhibition were determined by the Student *t* test. The results reported in the tables and figures are the mean ± the standard deviation (SD) for three to five experiments. The in vivo experiments were performed with groups of 10 mice.

RESULTS

With the intent of establishing a model of i.c. infection with *C. neoformans*, we performed experiments in which different doses of *C. neoformans* were injected i.c. into naive mice. The animals were examined for survival at 60 days, and the number of dead mice out of the total numbers of mice tested was recorded. As shown in Table 1, the mortality of the mice was strictly dose dependent. The median survival time (MST) ranged from 6.6 to 23.2 days with inocula of 10^7 to 10^3 yeast cells per mouse. Twenty-three of 30 mice died following i.c. injection of 10^3 yeast cells (24% of survivors), whereas doses of $\geq 10^4$ cells of *C. neoformans* resulted in no survival. In parallel experiments, *C. neoformans* growth in the brain and other organs was assessed by a CFU assay. Table 2 shows that the microorganisms gradually grew in the brain, which appeared heavily colonized 7 days after challenge. This phenomenon was followed by the subsequent appearance of CFU in the periphery. A histopathological analysis of brain sections from mice injected 15 days earlier with 10^3 yeast cells per mouse was performed. Figure 1A shows severe, diffuse meningitis with an extensive edematous reaction, characterized by numerous encapsulated yeast and mononuclear inflammatory cells (Fig. 1B). As depicted in Fig. 1C, large necrotic paraventricular areas were also present and consisted mainly of encapsulated yeast cells, although infiltrates with a few inflammatory cells were also evident (Fig. 1D).

TABLE 1. Survival of naive mice injected i.c. with various doses of *C. neoformans*

Dose (cells/mouse)	MST ± SD (range), in days	Mortality[a]
10^7	6.6 ± 0.8 (6–8)	40/40
10^6	9.2 ± 1.8 (6–16)	40/40
10^5	12.7 ± 3.7 (7–22)	40/40
10^4	21.1 ± 1.8 (10–30)	40/40
10^3	23.2 ± 3.7 (11–>60)	23/30

[a] Number of dead mice out of the total number of mice tested at 60 days.

To establish whether yeast opsonization could have any effect on cryptococcal pathogenicity, we incubated yeast cells in vitro with fresh mouse serum (20 min at 37°C) prior to i.c. challenge. Control mice received unopsonized *C. neoformans*. We found that the MST of mice receiving opsonized yeast cells (10^3 or 10^4 yeast cells per mouse) was significantly longer than that of mice receiving unopsonized yeast cells (Fig. 2). In contrast, *C. neoformans* opsonization did not affect the MST of mice receiving larger inocula (10^5 and 10^6 yeast cells per mouse).

Since the administration of chloroquine plus colchicine is known to affect the phagocytic compartment (5, 11, 13), we

TABLE 2. Kinetics of recovery of CFU from various organs of *C. neoformans*-challenged mice

Days after infection[a]	Mean CFU (10^3) recovered from[b]:			
	Brain	Spleen	Kidneys	Lungs
1	0.1	ND	ND	ND
3	2	ND	ND	ND
7	97	ND	ND	ND
10	667	0.5	ND	0.07
14	1,371	0.8	0.09	0.8
18	1,746	0.7	0.5	1.7
21	3,948	0.7	0.3	2.1

[a] Mice were injected i.c. with 10^3 *C. neoformans* cells. Groups of three mice were sacrificed at the indicated times after infection.

[b] SDs of less than 10% have been omitted. ND, not detectable.

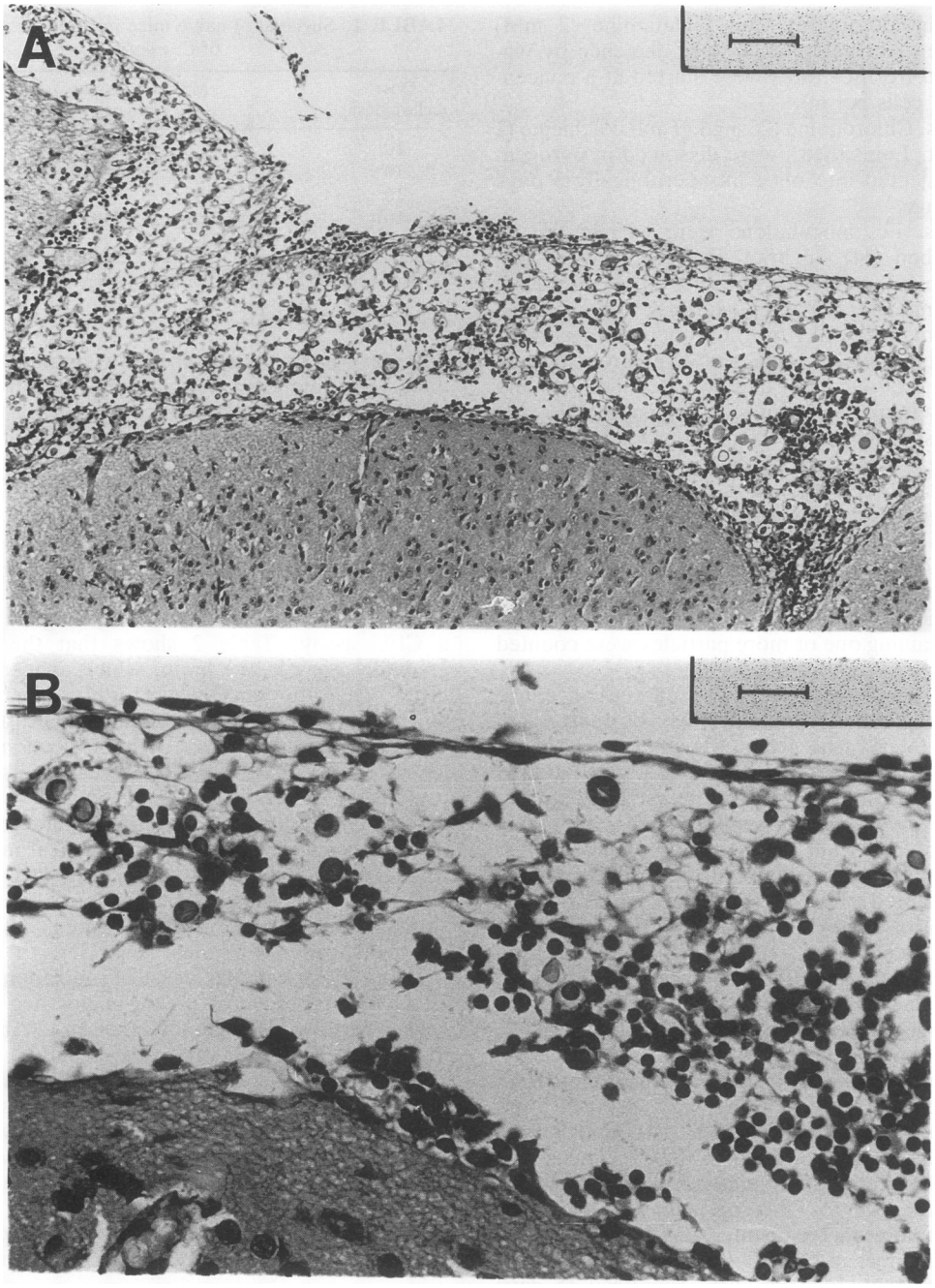

FIG. 1. Photomicrographs of parasagittal brain sections showing the effect of i.c. injection with *C. neoformans*. At 15 days after infection, a histopathological analysis of periodic acid-Schiff-stained brain sections was performed. Panel A shows meninges in which encapsulated yeast cells and an inflammatory reaction are evident, as detailed in panel B. Panel C shows necrotic paraventricular areas; details are shown in panel D. Bars: 30 μm (A and C); 120 μm (B and D).

questioned whether these drugs were effective in our experimental model. For this purpose, mice were inoculated intraperitoneally with chloroquine plus colchicine 5 days before challenge and then injected i.c. with various doses of *C. neoformans*. Figure 3 shows that drug-treated mice receiving 10^3 or 10^4 yeast cells per mouse had an MST significantly shorter than that of non-drug-treated mice. In contrast, at high doses (10^5 or 10^6 yeast cells per mouse), the MSTs of drug-treated and non-drug-treated mice were the same.

In other experiments, drug-treated animals were challenged i.c. with 10^4 opsonized or unopsonized yeast cells (Table 3). Opsonization resulted in a significant enhancement of the MST. In particular, MSTs were 22.4 and 12.1 days in mice challenged with opsonized or unopsonized yeast cells, respectively. While 100% of the animals died following injection with unopsonized *C. neoformans*, 24% of the animals survived a challenge with opsonized *C. neoformans*.

In an attempt to dissect the events involved in resistance

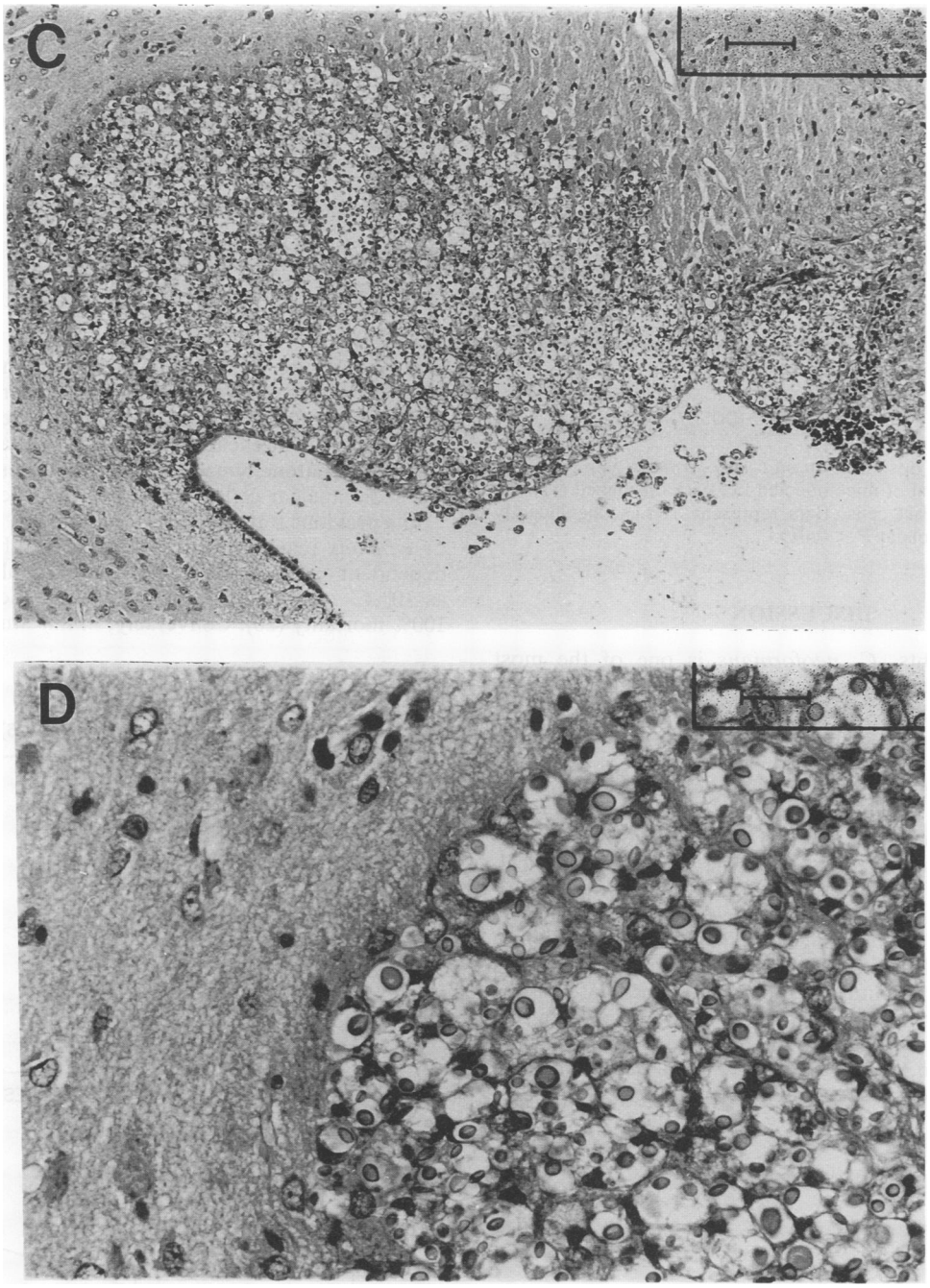

FIG. 1—*Continued.*

to i.c. infection with *C. neoformans*, we performed in vitro experiments using the recently established BV-2 microglial cell line (1). We evaluated the phagocytic properties of the microglial cells by incubating BV-2 cells with yeast cells that had or had not been opsonized. After 2 h, 51% of the BV-2 cells had ingested unopsonized yeast cells, whereas the percentage was 75% when opsonized *C. neoformans* microorganisms were used (Fig. 4A). Moreover, the number of yeast cells ingested per BV-2 cell was augmented by opsonization, as indicated by the increased phagocytosis index (Fig. 4A). Subsequently, BV-2 cell anticryptococcal ability was analyzed. Various concentrations of BV-2 cells were incubated with opsonized or unopsonized *C. neoformans* for 8 h at 37°C, and a CFU assay was performed. As reported in Fig. 4B, BV-2 cells were able to inhibit the CFU of opsonized *C. neoformans* in an effector-to-target cell ratio-dependent manner. Maximal levels of activity were reached at effector-to-target cell ratios of ≥100:1. In contrast, under the same conditions, BV-2 cells failed to exert any antifungal activity against unopsonized targets (Fig. 4B). When long-term experiments, in which the coincubation time was increased to at least 36 h, were performed, we were again able to detect significant levels of antifungal activity against unopsonized targets (data not shown).

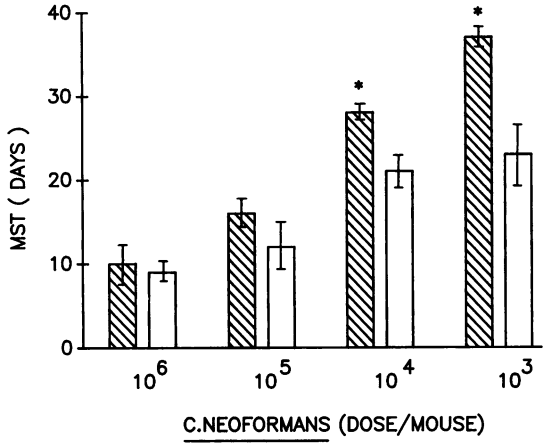

FIG. 2. Effect of opsonization on *C. neoformans* pathogenicity. Shown is the survival of mice injected i.c. with opsonized (▧) and unopsonized (□) yeast cells. Bars represent SDs. *, significantly different from controls at $P < 0.01$.

DISCUSSION

In AIDS patients, *C. neoformans* is one of the most common causes of life-threatening infections (4). Although the CNS represents a crucial target for this pathogen (21), scant information is available on the immune mechanisms involved in antifungal resistance within the CNS. Recently, Salkowski and Balish (35) demonstrated that immunocompetent and doubly (beige and athymic) immunodeficient mice differed in their susceptibilities to a systemic injection with *C. neoformans*. Interestingly, only the former showed cellular infiltrates or abscess formation in or near the cryptococcal foci within the brain. Nevertheless, the dissemination and rate of growth of the fungus in the brain were similar in both genotypes. This result suggests that brain mechanisms that control the onset and development of a local infection(s) are very peculiar. The present study describes a murine model of i.c. infection with *C. neoformans*. Following i.c. fungal inoculation, mice develop a lethal infection. The phenomenon is dose dependent and characterized by massive colonization of the brain. A histopathological analysis reveals the occurrence of generalized meningoencephalitis associated with an extensive edematous reaction. Necrotic areas with foci of encapsulated yeast cells are also observed within the parenchyma. Cellular infiltrates consisting of a few mononuclear cells are present near the foci.

In a previous report, we showed that i.c. administration of *C. albicans* results in a lethal infection, the severity being dependent on the inoculum size. In particular, doses as high as 10^6 *C. albicans* cells per mouse are needed to achieve 100% mortality (26). Conversely, in this study, all mice died

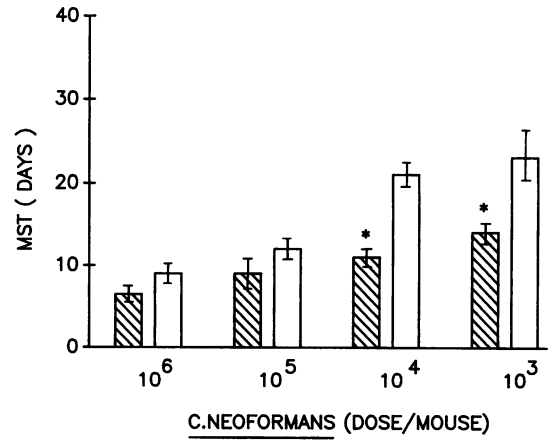

FIG. 3. Effect of chloroquine plus colchicine on the survival of mice challenged i.c. with various doses of *C. neoformans*. Symbols: ▧, drug-treated mice; □, non-drug-treated mice. Bars represent SDs. *, significantly different from controls at $P < 0.01$.

TABLE 3. Survival of drug-treated mice challenged with opsonized and unopsonized *C. neoformans*

C. neoformans opsonization[a]	MST ± SD (range), in days	Mortality[b]
No	12.1 ± 1.2 (4–18)	30/30
Yes	22.4[c] ± 2.38 (15–>60)	23/30

[a] Following 20 min of incubation with fresh mouse serum, *C. neoformans* was given as a single i.c. injection (10^4 yeast cells per mouse) to animals that had received chloroquine plus colchicine 5 days earlier.
[b] See Table 1, footnote *a*.
[c] Significantly different from controls at $P < 0.01$.

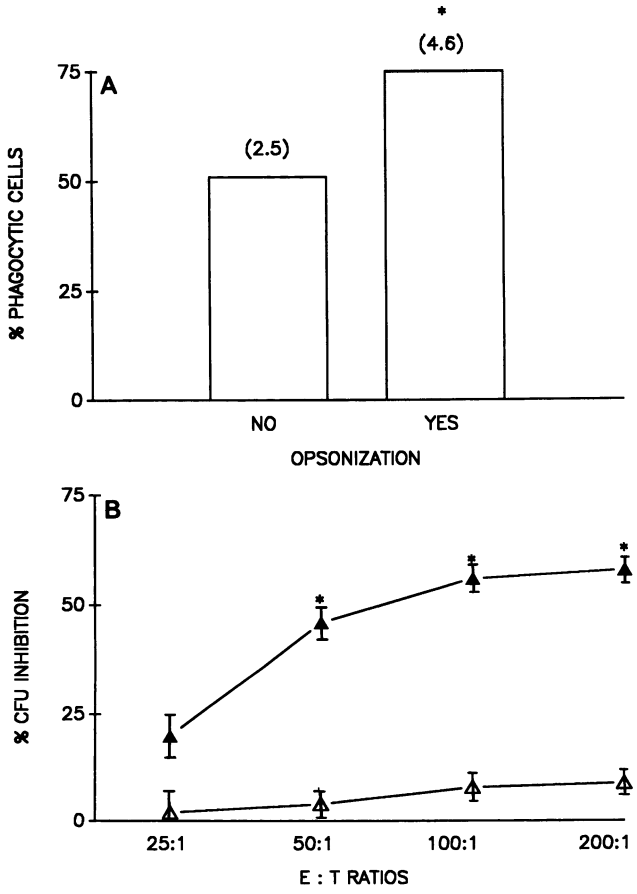

FIG. 4. Effect of opsonization on phagocytic and anticryptococcal activities of BV-2 microglial cells. (A) Percentage of phagocytic cells and phagocytosis index (in parentheses). (B) Percentage of CFU inhibition by BV-2 cells of opsonized (▲) and unopsonized (△) yeast cells. Bars represent SDs. *, significantly different from controls at $P < 0.01$. E:T, effector-to-target cell.

following i.c. injection with 10^4 *C. neoformans* cells per mouse (Table 1). We cannot ascribe such a difference to differences in virulence between the fungi, in that, when they are given systemically, opposite results are observed, i.e., mice are more susceptible to *C. albicans* than to *C. neoformans* (data not shown). Thus, we conclude that the cerebral district is more susceptible to *C. neoformans* than to *C. albicans*, and we hypothesize that in the brain compartment there are effector cells less active against the former fungus. Preliminary experiments support this hypothesis. In fact, using in vivo local transfer of microglial cells, we consistently failed to protect mice against i.c. infection with *C. neoformans* (data not shown). Conversely, such a procedure is known to be highly effective against *C. albicans* infection (2). Therefore, microglial cells are crucial elements of defense against *C. albicans* but are not as efficient against *C. neoformans*. Nevertheless, phagocytic cells play a defensive role against i.c. infection with *C. neoformans*, as shown by our studies of drug-treated mice. Previous reports demonstrated that in vivo administration of colchicine and/or chloroquine affects the phagocytic compartment by blocking phagocytosis, endocytosis, and secretory functions (5, 11, 13). Furthermore, by monitoring local cellular responses to a penetrating brain injury, Giulian et al. (13) showed that the number of mononuclear phagocytes at injury sites is significantly reduced and that astrogliosis is inhibited in drug-treated animals compared with controls. Thus, a major effect of chloroquine and colchicine on myelomonocytic cells has been proven, although the possibility that they act on other cell types cannot be excluded. In this study, treatment with chloroquine plus colchicine significantly reduced the life spans of mice inoculated i.c. with *C. neoformans* compared with those of non-drug-treated mice. When the size of the *C. neoformans* inoculum was reduced, the immunosuppressive effects of the drugs were more evident. This finding strongly supports the possibility that drug-susceptible, phagocyte-mediated mechanisms are important in resistance to *C. neoformans* within the CNS. Although it would be difficult to fully establish the extent of inhibition and the spectrum of action of chloroquine and colchicine at the cerebral level, this model may provide a useful tool for dissecting the anticryptococcal mechanisms involved in local resistance to i.c. fungal infections.

Clinical and experimental evidence indicates that initial pulmonary defenses against cryptococcosis involve T cells (18) and alveolar macrophages (24, 37). Recent studies suggest that natural killer cells may contribute to natural immunity against fungi (16, 17, 30, 32), while PMN, monocytes, and macrophages are likely crucial defenses against cryptococcemia. Killing of *C. neoformans* by PMN and monocytes occurs via intra- and extracellular mechanisms, while opsonization is important in enhancing the efficacy of anticryptococcal effector cells (14, 19, 21). In agreement with these reports, our in vivo experiments revealed augmented life spans in mice challenged i.c. with opsonized *C. neoformans* compared with mice receiving unopsonized fungi. Similar results were observed for drug-treated mice, suggesting that anticryptococcal elements other than phagocytic cells are effective against opsonized fungi. Some residual phagocyte-mediated activity, still present in drug-treated animals, may have accounted for the enhanced MST. In any case, our results imply that opsonization renders *C. neoformans* more susceptible to fungicidal brain effector cells.

Our in vitro studies with the microglial cell line revealed that phagocytosis is increased when opsonized fungi are used, whereas antifungal activity occurs only against opsonized targets, unless long-term coincubation times are used. These data provide two major conclusions: (i) microglial cells are active anticryptococcal elements, in vitro and (ii) opsonization is also an important event in the fulfillment of microglia-mediated anticryptococcal activity in vitro.

Overall, our experimental model of i.c. infection may be a useful tool in further studies aimed at clarifying the cellular and molecular mechanisms controlling the CNS immune response during a *C. neoformans* infection.

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