

## Chloroquine induces human mononuclear phagocytes to inhibit and kill *Cryptococcus neoformans* by a mechanism independent of iron deprivation.

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### Research Article

Infections due to *Cryptococcus neoformans* are common in AIDS patients. We investigated the effect of chloroquine, which raises the pH of phagolysosomes, on the anticryptococcal activity of mononuclear phagocytes. *C. neoformans* multiplied within monocyte-derived macrophages (MDM) in the absence of chloroquine but were killed with the addition of chloroquine. Ammonium chloride was also beneficial, suggesting that effects were mediated by alkalinizing the phagolysosome. Chloroquine inhibits growth of other intracellular pathogens by limiting iron availability. However, chloroquine-induced augmentation of MDM anticryptococcal activity was unaffected by iron nitriloacetate, demonstrating that chloroquine worked by a mechanism independent of iron deprivation. There was an inverse correlation between growth of *C. neoformans* in cell-free media and pH, suggesting that some of the effect of chloroquine on the anticryptococcal activity of MDM could be explained by relatively poor growth at higher pH. Chloroquine enhanced MDM anticryptococcal activity against all tested cryptococcal strains except for one large-capsule strain which was not phagocytosed. Positive effects of chloroquine were also seen in monocytes from both HIV-infected and -uninfected donors. Finally, chloroquine was therapeutic in experimental cryptococcosis in outbred and severe combined immunodeficient mice. Thus, chloroquine enhances the activity of mononuclear phagocytes against *C. neoformans* by iron-independent, pH-dependent mechanisms and is therapeutic in murine models of cryptococcosis. Chloroquine might have clinical utility for the prophylaxis and treatment of human cryptococcosis.

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# Chloroquine Induces Human Mononuclear Phagocytes to Inhibit and Kill *Cryptococcus neoformans* by a Mechanism Independent of Iron Deprivation

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## Abstract

Infections due to *Cryptococcus neoformans* are common in AIDS patients. We investigated the effect of chloroquine, which raises the pH of phagolysosomes, on the anticryptococcal activity of mononuclear phagocytes. *C. neoformans* multiplied within monocyte-derived macrophages (MDM) in the absence of chloroquine but were killed with the addition of chloroquine. Ammonium chloride was also beneficial, suggesting that effects were mediated by alkalinizing the phagolysosome. Chloroquine inhibits growth of other intracellular pathogens by limiting iron availability. However, chloroquine-induced augmentation of MDM anticryptococcal activity was unaffected by iron nitriloacetate, demonstrating that chloroquine worked by a mechanism independent of iron deprivation. There was an inverse correlation between growth of *C. neoformans* in cell-free media and pH, suggesting that some of the effect of chloroquine on the anticryptococcal activity of MDM could be explained by relatively poor growth at higher pH. Chloroquine enhanced MDM anticryptococcal activity against all tested cryptococcal strains except for one large-capsule strain which was not phagocytosed. Positive effects of chloroquine were also seen in monocytes from both HIV-infected and -uninfected donors. Finally, chloroquine was therapeutic in experimental cryptococcosis in outbred and severe combined immunodeficient mice. Thus, chloroquine enhances the activity of mononuclear phagocytes against *C. neoformans* by iron-independent, pH-dependent mechanisms and is therapeutic in murine models of cryptococcosis. Chloroquine might have clinical utility for the prophylaxis and treatment of human cryptococcosis. (*J. Clin. Invest.* 1997. 100:1640–1646.)  
Key words: chloroquine • macrophage • HIV • Cryptococcus • iron

## Introduction

Infections due to the yeast *Cryptococcus neoformans* are a common cause of morbidity and mortality in persons infected with HIV (1–3). Even when successfully treated, AIDS patients with cryptococcosis are not cured and require lifetime

therapy to prevent relapse. Thus, new therapeutic agents for treatment and prophylaxis of cryptococcal infections are needed. Ideal agents would have low toxicity, be relatively inexpensive, be easy to administer, and have a spectrum of activity that encompasses a range of pathogens commonly seen in individuals with HIV.

Chloroquine, a diprotic weak base which raises the endocytic and lysosomal pH of eukaryotic cells (4), is a widely used, relatively safe drug for the treatment of malaria, RA, and SLE. Its long half-life (~4 d) in humans allows weekly administration. In human mononuclear phagocytes, chloroquine inhibits the growth of *Legionella pneumophila*, *Histoplasma capsulatum*, and *Francisella tularensis* by limiting iron availability in the phagolysosome (4–6). This is accomplished because mobilization of iron from transferrin and ferritin, the two major sources of iron in the mononuclear phagocyte, is dependent upon an acidic environment. Iron dissociates from transferrin at acid pH, becoming available to the microbe (4, 7). Thus, both ferric ions are released from transferrin at pH 5 or below, and one ferric ion is released at pH 6. At pH  $\geq 7$ , ferric ions remain bound to transferrin. Iron availability from ferritin is thought to occur after pH-dependent proteolysis in lysosomes (8–10). The ability of chloroquine to inhibit the growth of *L. pneumophila* and *H. capsulatum* in human mononuclear phagocytes is reversed by ferric nitrilotriacetate (FeNTA,<sup>1</sup> an iron chelate that freely enters cells and releases iron independent of pH over the range of pH 5–8) but not holotransferrin (which releases iron only in an acidic environment) (4, 5, 11, 12). In addition to its beneficial effects in vitro, chloroquine proved therapeutic in a murine model of histoplasmosis (5).

Based on these studies, we hypothesized that chloroquine would similarly enhance the anticryptococcal capacity of human mononuclear phagocytes. We found that chloroquine did have a pronounced beneficial effect, although the mechanism was independent of iron deprivation and apparently mediated, at least in part, by the relatively poor growth of *C. neoformans* at higher pH.

## Methods

**Materials.** Unless otherwise noted, reagents were obtained from Sigma Chemical Co. (St. Louis, MO). All experiments were performed under conditions carefully designed to minimize endotoxin contamination, as described (13). RPMI 1640 and PBS were obtained from BioWhittaker, Inc. (Walkersville, MD) and contained < 0.005 endotoxin U/ml. Pooled human serum (PHS) was obtained by combining serum from  $\geq 10$  healthy donors under ice-cold conditions and

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1. **Abbreviations used in this paper:** FeNTA, ferric nitriloacetate; MDM, monocyte-derived macrophages; NTA, nitriloacetate; PHS, pooled human serum; RITC, rhodamine isothiocyanate; SCID, severe combined immunodeficiency.

storing it at  $-70^{\circ}\text{C}$  to preserve complement activity. PHS contained undetectable levels of endotoxin as measured by the Limulus lysate assay. Culture medium was RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% PHS. NTA and FeNTA were prepared fresh for each experiment, as described, and filter-sterilized before use (4, 11). Unless otherwise noted, cells were incubated at  $37^{\circ}\text{C}$  in humidified air supplemented with 5%  $\text{CO}_2$ .

**C. neoformans.** Serotype A strains 6, 15, 98, 110, and 145 were gifts of Dr. Thomas Mitchell (Duke University, Durham, NC) (14). Strain ME was obtained from a patient at Boston Medical Center with cryptococcal meningitis (15). Except where otherwise noted, strain 145 was used for all studies presented herein. *C. neoformans* was grown on Sabouraud dextrose agar at  $30^{\circ}\text{C}$ . Fungi were harvested after 4 d of growth, washed three times in PBS, counted using a hemocytometer, and resuspended at the desired concentration.

**Isolation of PBMCs.** Peripheral blood was obtained by venipuncture from healthy volunteers recruited from the staff, students, and faculty at Boston University Medical Center. The three HIV-seropositive donors were recruited from the immunodeficiency clinics at Boston Medical Center and had CD4 counts of 40, 80, and 150 cells/ $\mu\text{l}$  blood. Blood donors did not have an acute concurrent illness or a history of cryptococcosis, and were not taking systemic antifungal medication or hematopoietic growth factors. Two of the three patients were taking antiretroviral treatment, although none was on protease inhibitors. The same donor was not used more than once.

Blood was anticoagulated with pyrogen-free heparin (Elkins-Sinn Inc., Cherry Hill, NJ) and centrifuged at 500 g for 15 min. PBMCs were isolated by centrifugation of the leukocyte-rich buffy-coat cells on a Ficoll-Hypaque density gradient. PBMCs were washed twice in PBS and then enriched for monocytes by depletion of T cells by rosetting with neuraminidase-treated sheep red blood cells. Previous studies from our laboratory using this technique have demonstrated that  $\sim 50\%$  of the leukocytes from both HIV-seropositive and -seronegative donors stain positively for the monocyte markers CD14 and/or CD13 by flow cytometric analysis. The remainder of the cells were mostly B and natural killer cells, with  $< 3\%$  T cells (16). These cells are hereafter referred to as monocytes. Monocyte-derived macrophages (MDM) were obtained by culturing monocytes for 7 d in half-area flat-bottomed wells containing 100  $\mu\text{l}$  culture medium. MDM were washed three times with RPMI 1640 before assay.

**Assay of antifungal activity.** Antifungal activity was determined as previously described (17, 18). Monocytes or MDM ( $2 \times 10^5$ ) and *C. neoformans* ( $10^4$  CFU) were incubated in wells of 96-well plates containing 100  $\mu\text{l}$  of culture medium supplemented with 10% (final concentration) PHS. Where indicated, chloroquine, NTA, FeNTA, holotransferrin, apotransferrin, and/or ammonium chloride, were added 30 min before the addition of *C. neoformans*. Plates were incubated for 18 h, after which effector cells were lysed with 0.1% Triton X-100, and the number of CFU of *C. neoformans* was determined by dilution and spread plates on Sabouraud dextrose agar. This concentration of Triton X-100 had no effect on fungal viability. For each assay, sets of wells containing *C. neoformans*, medium, and PHS (but no effector cells) were incubated at  $4^{\circ}\text{C}$  to determine the inoculum of live organisms. We have found that replication of *C. neoformans* does not occur after incubation at  $4^{\circ}\text{C}$  for 18 h. Triton X-100 was added to the  $4^{\circ}\text{C}$  control wells as well as to the experimental wells before plating. Results are expressed as percent growth according to the formula,  $\{(\text{experimental CFU}/\text{inoculated CFU}) - 1\} \times 100$ . Thus, a value of zero indicates no net fungal growth. Values of 100, 300, and 700% would indicate an average of 1, 2, and 3 replications, respectively. A negative value indicates that the number of CFU decreased during the incubation period, and therefore that some fungal killing had occurred.

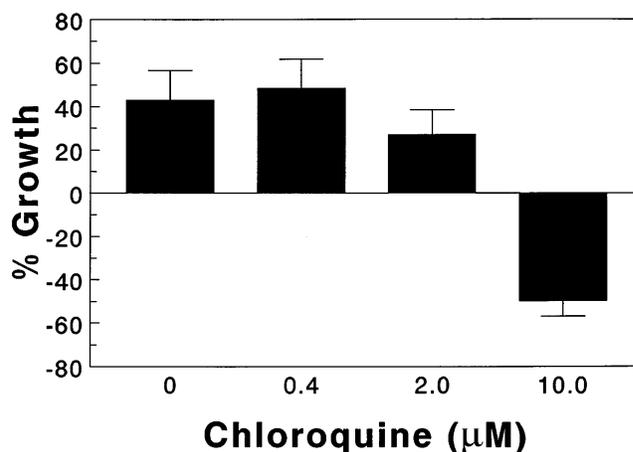
**Binding assay.** Binding of *C. neoformans* to MDM was determined using a modification of our previously described assay (19). MDM were incubated with *C. neoformans* in the presence or absence of chloroquine, using the exact same conditions as for the above antifungal activity except that the *C. neoformans* were labeled with rhodamine isothiocyanate (RITC) to facilitate their identification. After

4 h of incubation, the percentage of fungi that were cell-associated (either surface-attached or internalized) was determined by epifluorescent microscopy. At least 200 yeast cells were counted per well.

**Phagocytosis assay.** Our previously described Uvitex assay was used to determine the percentage of cell-associated fungi that were internalized (phagocytosed) by the MDM (19, 20). Briefly, MDM and RITC-labeled *C. neoformans* were coincubated for 4 h as described for the binding assay. Unbound organisms were washed free, and the cells were incubated with 0.1% Uvitex 2B (also known as Fungical A and diaethanol; Specialty Chemicals for Medical Diagnostics, Kandern, Germany) for 1 min. The wells were washed, fixed, and examined under an epifluorescent microscope. Internalized organisms stain with RITC alone, whereas extracellular organisms stain with both RITC and Uvitex. At least 100 cell-associated *C. neoformans* were scored per well.

**Murine model of systemic cryptococcosis.** 4–5-wk-old BALB/cByj-Smn-Prkdc scid (severe combined immunodeficiency, SCID) female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 3–4-wk-old male outbred ICR mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The mice were housed in microisolator cages and adapted to their environment before experimentation. Chloroquine was prepared in PBS, filter-sterilized, and injected intraperitoneally in a total volume of 200  $\mu\text{l}$ . Control mice received 200  $\mu\text{l}$  PBS alone. A suspension of *C. neoformans* strain 145 was prepared in PBS as described above and injected intravenously in a total volume of 100  $\mu\text{l}$ . Fungal inocula used were based on preliminary studies (data not shown) and previously described models of cryptococcosis (21).

**Statistics.** Except as noted below, means and SE were compared using the two-tailed paired *t* test. Adjustments for significance based on multiple comparisons were made using Bonferroni's method. Statistical significance was considered to be achieved if the *P* value multiplied by the number of comparisons was  $< 0.05$ . Linear regression was used for experiments comparing fungal growth with pH of culture medium. The Mann-Whitney rank sum test was used to compare median survival in the mouse experiments. All statistical calculations were performed using SigmaStat Statistical Software (Jandel Scientific, San Rafael, CA).



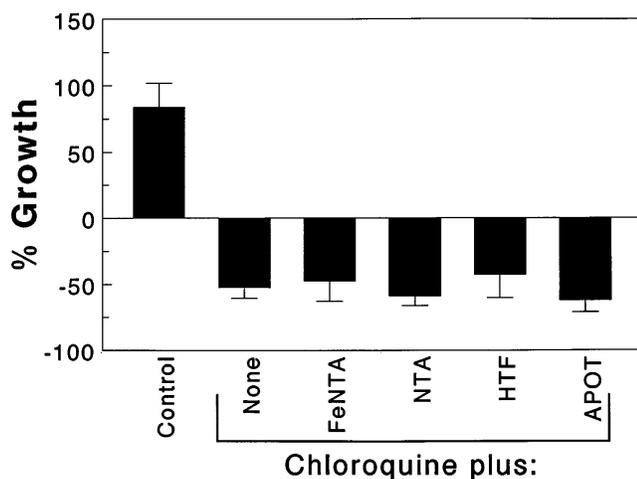
**Figure 1.** Dose-response effects of chloroquine on the activity of MDM against *C. neoformans*. MDM and *C. neoformans* were incubated for 18 h in the presence of the indicated concentration of chloroquine, after which antifungal activity was assayed as described in Methods. Data are means  $\pm$  SE of four separate experiments, each of which was performed in triplicate.  $P < 0.0001$  comparing no chloroquine with chloroquine at 10  $\mu\text{M}$ .  $P = 0.059$  comparing no chloroquine with chloroquine at 2  $\mu\text{M}$ .

## Results

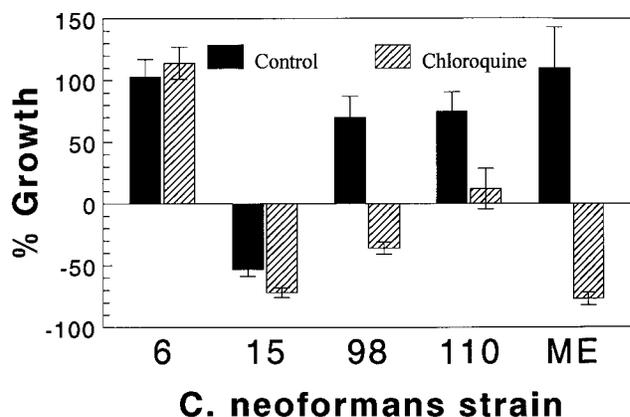
*Effect of chloroquine and ammonium chloride on activity of MDM against C. neoformans.* In initial experiments, the effect of varying concentrations of chloroquine on the anticytotoxic activity of MDM was assayed (Fig. 1). Consistent with published data from our laboratory and others (22, 23), in the absence of chloroquine, cryptococcal growth occurred in MDM. However, when 10  $\mu$ M chloroquine was present during the incubation, fungi were killed by MDM. Lower concentrations of chloroquine were not effective. If the incubation period of MDM and *C. neoformans* was shortened from 18 to 2 h, the addition of 10  $\mu$ M chloroquine was without effect (data not shown). Consistent with data from others (24), chloroquine at concentrations up to 10  $\mu$ M had no direct effect on the growth of *C. neoformans* in culture medium without MDM (data not shown).

The antifungal activity of MDM was also enhanced in the presence of the weak base, ammonium chloride. In three independent experiments (each performed in triplicate), percent growth was  $45.7 \pm 12.9$  and  $3.6 \pm 6.0$  (means  $\pm$  SE;  $P = 0.002$ ) in the absence and presence, respectively, of 20 mM ammonium chloride. Like chloroquine, ammonium chloride is a weak base which has been shown to raise the pH of lysosomal compartments (4, 25).

*Contribution of iron deprivation to the effects of chloroquine.* As discussed above, chloroquine inhibits the growth of *L. pneumophila* and *H. capsulatum* by limiting iron availability in the phagolysosome, an effect that is reversed by FeNTA but not holotransferrin (4, 5). Accordingly, in the next set of experiments we examined whether FeNTA and holotransferrin could reverse the effects of chloroquine on the anticytotoxic



**Figure 2.** The effects of chloroquine are independent of iron deprivation. MDM were incubated for 18 h with *C. neoformans* strain 145 in the absence (Control) or presence of 10  $\mu$ M chloroquine. Groups that received chloroquine also received no additions (None), 50  $\mu$ g/ml FeNTA, 50  $\mu$ g/ml NTA, 10 mg/ml holotransferrin (HTF), or 10 mg/ml apotransferrin (APOT). Wells were incubated for 18 h, after which antifungal activity was assayed as described in Methods. Data are means  $\pm$  SE of four separate experiments, each of which was performed in triplicate. There were no significant differences between any of the chloroquine-containing groups.  $P < 0.0001$  comparing no chloroquine with any group containing chloroquine.



**Figure 3.** Strain-related variation in the effect of chloroquine on activity of MDM against *C. neoformans*. MDM were incubated for 18 h with the indicated strain of *C. neoformans* in the absence (Control) or presence of 10  $\mu$ M chloroquine, after which antifungal activity was assayed as described in Methods. Data are means  $\pm$  SE of two to three separate experiments, each of which was performed in triplicate.  $P < 0.005$  comparing no chloroquine with chloroquine for all of the strains except strain 6.

coccal activity of MDM. The iron-unsaturated forms of these compounds (NTA and apotransferrin) were included as controls. As in the experiments presented above, 10  $\mu$ M chloroquine significantly augmented the anticytotoxic activity of MDM (Fig. 2). FeNTA, NTA, holotransferrin, and apotransferrin had no significant effect on the anticytotoxic activity of chloroquine-treated MDM, strongly suggesting that chloroquine's effects are independent of iron deprivation. FeNTA, NTA, holotransferrin, and apotransferrin also had no significant effects on growth of *C. neoformans* in wells containing MDM but no chloroquine (data not shown).

*Strain-related variation in the effect of chloroquine on activity of MDM against C. neoformans.* The above experiments established the beneficial effects of chloroquine on the anticytotoxic activity of MDM against *C. neoformans* strain 145. Because previous studies have demonstrated strain-related variations in certain responses of phagocytes to *C. neoformans* (14, 15, 26, 27), we next studied the activity of MDM against five additional strains of *C. neoformans*. Chloroquine enhanced the anticytotoxic activity of MDM against all of the strains tested except for strain 6 (Fig. 3). This strain is unique among the strains studied in that it is heavily encapsulated when grown on Sabouraud dextrose agar (14).

*The effect of pH on cryptococcal growth.* The above experiments established that chloroquine augments the antifungal activity of human MDM by a mechanism independent of iron deprivation. As it is known that chloroquine alkalizes the phagolysosome, we hypothesized that the beneficial effects of chloroquine on MDM-mediated anticytotoxic activity are, at least in part, a direct result of the inability of *C. neoformans* to grow well at neutral and alkaline pH compared with acidic pH. Accordingly, cryptococcal growth in cell-free tissue culture medium as a function of pH was determined for strains 145 and 6 (Fig. 4). The pH of the culture medium was adjusted with hydrochloric acid or sodium hydroxide, and remained within 0.2 pH U for the duration of the 18-h incubation period.

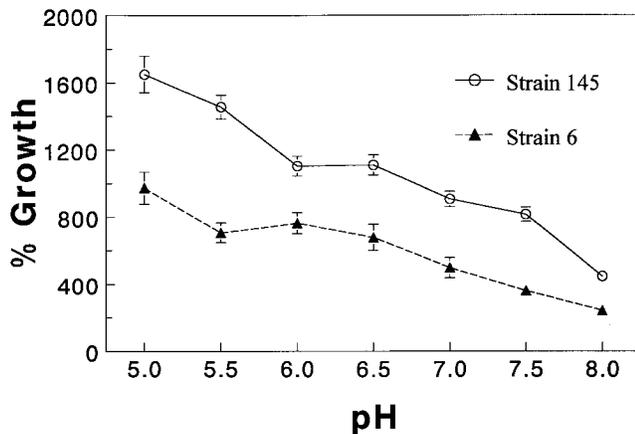


Figure 4. Effect of pH on growth of cryptococcal strains. Strains 145 and 6 were incubated in tissue culture medium adjusted to the indicated pH for 18 h, and percent growth was measured, as described in Methods. There was a significant inverse correlation between pH and percent growth ( $P < 0.001$  for both strains by linear regression). Data are means  $\pm$  SE of two separate experiments, each of which was performed in triplicate.

For both strains tested, there was a significant inverse correlation between percent growth and pH, with approximately fourfold greater growth seen at pH 5.0 than at pH 8.0.

**Effect of chloroquine on binding and phagocytosis.** As demonstrated in Figs. 3 and 4, despite an inverse correlation between growth and pH, chloroquine failed to promote the antifungal activity of MDM against strain 6. In contrast, chloroquine had a beneficial effect on all other tested strains. Strain 6 is a heavily encapsulated strain, and cryptococcal capsule is known to be antiphagocytic (28). Thus, we hypothesized that strain 6, because of its heavy encapsulation, does not get phagocytosed effectively by MDM. Chloroquine increases the pH of intracellular compartments without having a significant effect on the pH of the extracellular culture medium. Thus,

pH-dependent effects of chloroquine on growth of *C. neoformans* would be expected to be limited to intracellular yeast cells. Accordingly, we next measured MDM-mediated binding and phagocytosis (internalization) of strains 145 and 6 in the presence and absence of chloroquine (Fig. 5). Chloroquine had no effect on binding and phagocytosis of either strain. For both strains, most of the *C. neoformans* added to the wells became cell-associated over the course of the 4-h assay, although there was a trend towards greater binding of strain 145 than strain 6. However, profound differences were seen when phagocytosis was examined: whereas  $> 80\%$  of cell-associated strain 145 were phagocytosed by the MDM,  $< 20\%$  of cell-associated strain 6 were actually internalized ( $P < 0.0001$ ). The majority of yeast cells from strains 15, 98, 110, and ME were internalized by MDM (data not shown).

**Effect of chloroquine on monocytes from HIV-seronegative and -seropositive donors.** The next set of experiments was designed to answer two basic questions. First, does chloroquine work on monocytes in addition to MDM? Second, as most cases of cryptococcosis occur in the setting of HIV infection, does chloroquine have a beneficial effect on monocytes from HIV-infected donors? Accordingly, PBMCs were isolated from both HIV-seropositive and -seronegative donors and tested for anticryptococcal activity in the presence and absence of chloroquine (Fig. 6). Similar to its effects on MDM, chloroquine augmented the anticryptococcal capacity of freshly isolated monocytes. Beneficial effects were seen regardless of the HIV status of the blood donor.

**Effect of chloroquine in murine models of cryptococcosis.** The final set of experiments examined the effect of chloroquine on mice experimentally infected with *C. neoformans*. Two strains of mice were selected for study, immunocompetent (outbred) and immunodeficient (SCID), based on the types of patient populations that get cryptococcosis. Mice received 15 mg/kg of chloroquine every other day. This dosage was based on preliminary studies which determined that mice receiving doses  $\geq 30$  mg/kg/d sometimes developed lethal toxicity from the chloroquine (data not shown). In both strains of mice, chloroquine was protective (Fig. 7).

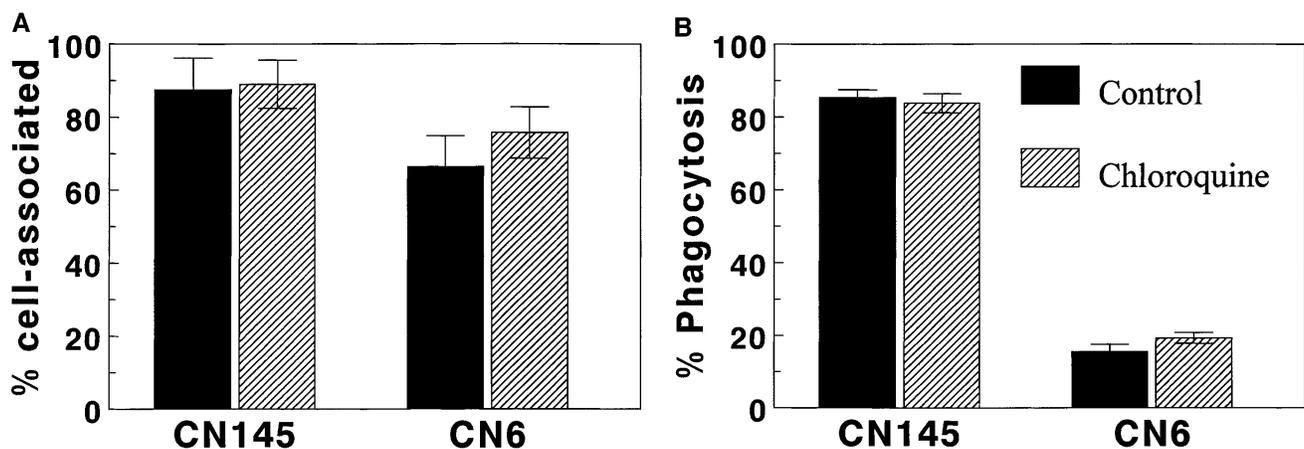


Figure 5. Binding and phagocytosis of strains 145 and 6. MDM were incubated for 4 h with either strain 145 or strain 6 of *C. neoformans* in the absence or presence of 10  $\mu$ M chloroquine, and then binding (cell-association) and phagocytosis (internalization) were determined as described in Methods. (A) Percentage of *C. neoformans* which was cell-associated. (B) Percentage of cell-associated *C. neoformans* that was actually phagocytosed (internalized).  $P < 0.0001$  comparing phagocytosis of the two strains regardless of the presence of chloroquine. Results are means  $\pm$  SE of two separate experiments, each of which was performed in triplicate.

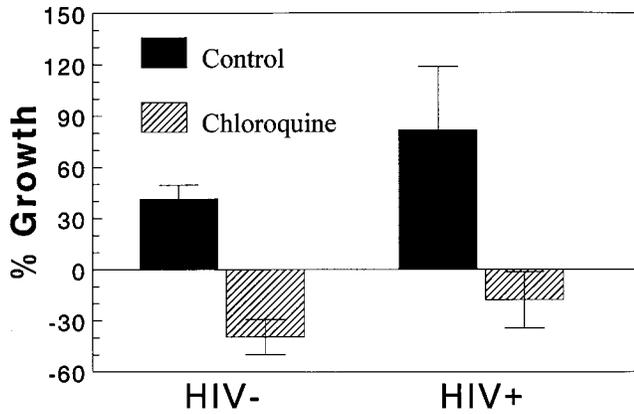


Figure 6. Effect of chloroquine on the anticytotoxic activity of monocytes from HIV-seronegative (HIV<sup>-</sup>) and seropositive (HIV<sup>+</sup>) donors. PBMCs from HIV<sup>-</sup> and HIV<sup>+</sup> donors were incubated for 18 h with *C. neoformans* strain 145 in the absence (Control) or presence of chloroquine, after which antifungal activity was assayed as described in Methods. Data are means  $\pm$  SE of three separate experiments, each of which was performed in triplicate.  $P < 0.001$  comparing HIV<sup>-</sup> in the absence and presence of chloroquine.  $P = 0.005$  comparing HIV<sup>+</sup> in the absence and presence of chloroquine.

## Discussion

The data presented demonstrate that chloroquine enhances the activity of freshly isolated monocytes and MDM against *C. neoformans*. Beneficial effects were seen in both HIV-seronegative and -seropositive donors. Moreover, chloroquine was therapeutic in two murine models of cryptococcosis. Taken together, these data suggest that chloroquine has potential as a novel therapeutic agent against cryptococcosis.

The inability of FeNTA to reverse the effects of chloroquine on the anticytotoxic activity of MDM argues strongly against a role for iron deprivation as a mechanism of action. *C. neoformans* is not known to produce siderophores, and its

mechanism of iron acquisition is uncertain (29, 30). There is experimental evidence that *C. neoformans* can generate ferrous iron at the cell surface via reduction of ferric chelates, with the subsequent acquisition of the ferrous iron (30). However, chloroquine enhances the activity of MDM against *L. pneumophila* (which also has an iron reductase system; reference 31) by pH-dependent iron deprivation (4). Recently, a transferrin-like molecule with iron-binding properties has been purified from *C. neoformans* and postulated to play a role in iron uptake by this organism (32). Regardless of the mechanism of iron acquisition, chloroquine and its effects on pH do not appear to affect the ability of *C. neoformans* to obtain adequate iron in the MDM phagolysosome.

pH did have a significant, direct effect on growth of *C. neoformans* in cell-free culture medium. Howard, using 2% buffered proteose peptone medium, also found that the growth of *C. neoformans* is markedly inhibited in media with initial pH values slightly above neutrality (33). The ability of *C. neoformans* to grow better at acidic pH may give it a survival advantage in the phagolysosome and help explain the observation that under certain conditions *C. neoformans* grows as well if not better inside MDM than in tissue culture medium (22, 23).

Our data suggest that at least some of the effect of chloroquine on the activity of MDM against *C. neoformans* can be explained by the relatively poor growth of this fungus at higher pH. However, even at pH 8.0, *C. neoformans* still grew in cell-free culture medium, albeit slowly, whereas chloroquine treatment resulted in MDM developing fungicidal properties. Slowing down the growth of the organism could give time to enable the fungicidal mechanisms of the MDM to take effect. Such mechanisms as they apply to *C. neoformans* are unknown, although the MDM respiratory burst does not appear to be essential (our unpublished data). Human mononuclear phagocytes are known to possess several neutral proteinases (including elastase, cathepsin G, at least two collagenases, and stromelysin), some of which are found in markedly increased amounts in MDM compared with monocytes (34, 35). This raises the possibility that in addition to slowing cryptococcal growth, raising the pH could result in more efficient antifungal

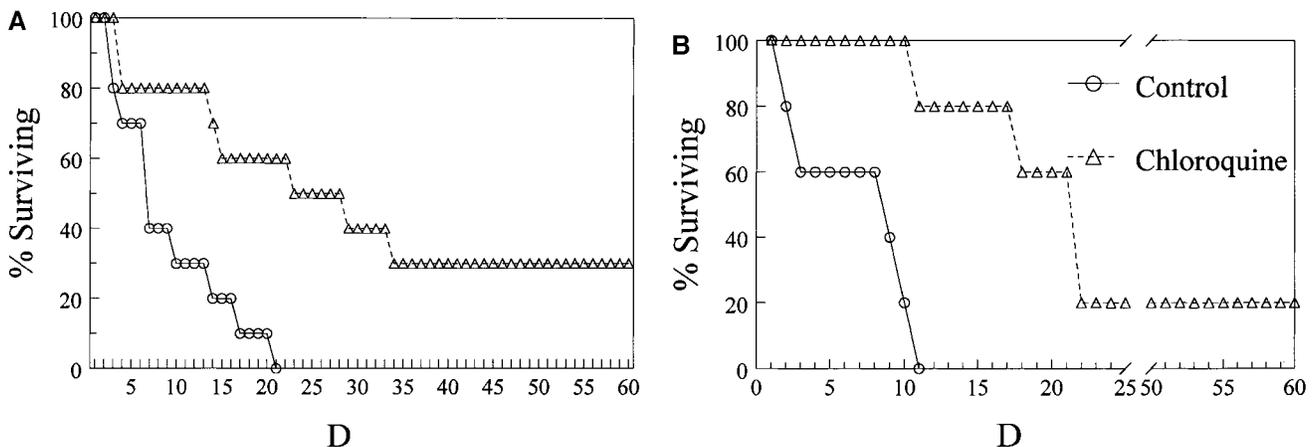


Figure 7. Effect of chloroquine on survival of mice with experimental cryptococcosis. Outbred (10 per group, A) and SCID (5 per group, B) mice were treated with 15 mg/kg of chloroquine intraperitoneally every other day beginning 2 d before infection. Control mice received PBS alone. Mice were injected intravenously with  $3 \times 10^6$  (outbred) or  $10^5$  (SCID) *C. neoformans* strain 145, and survival was determined daily.  $P = 0.02$  and 0.008 comparing median survival of chloroquine and control groups by Mann-Whitney rank sum test for outbred and SCID mice, respectively.

activity by optimizing the pH of certain antimicrobial MDM enzymes. If so, this would be in contradistinction to the situation with other intracellular microbes studied, where acidification is thought to enhance antimicrobial activity of phagocytes, in part because many hydrolytic enzymes are active only at low pH (36).

Chloroquine has also been shown to inhibit secretion of certain immunoactive substances, including TNF- $\alpha$  and PGE<sub>2</sub> (37, 38). Other biological effects of chloroquine have been described that are purportedly pH-independent, including inhibition of calcium release from macrophage intracellular stores and intercalation into DNA (39). However, ammonium chloride also enhanced the anticryptococcal activity of MDM. As both ammonium chloride and chloroquine raise the pH of phagolysosomes (4, 25), these findings strongly suggest that the effects of chloroquine on MDM activity against *C. neoformans* are mediated by an increase in the pH of the phagolysosome. The potency of ammonium chloride was less than that of chloroquine, a finding that could be due to the capacity of ammonium chloride (but not chloroquine) to inhibit phagolysosome fusion (40).

Chloroquine had beneficial effects on all *C. neoformans* strains tested except for strain 6, which is a heavily encapsulated strain that was poorly phagocytosed by MDM. This finding is consistent with the hypothesis that, because chloroquine works on intracellular compartments, internalization of *C. neoformans* is necessary for chloroquine's activity. The medical correlate of this finding is that in clinical cases of cryptococcosis, where both intracellular and extracellular fungi are seen (41), chloroquine would only be expected to have effects on the intracellular pool of fungi. Theoretically, then, administration of anticryptococcal antibody (which would be expected to increase phagocytosis of *C. neoformans*) should work synergistically in combination with chloroquine. Clinical trials of a humanized anticryptococcal mAb have been proposed (42).

Chloroquine was protective in two distinct murine models of cryptococcosis, one that used immunocompetent outbred mice and the other that used SCID mice. These two mouse strains were chosen because they serve as models for most human hosts with cryptococcosis (1–3, 21). Thus, the outbred mice mimic the clinical situation of cryptococcosis in the immunocompetent patient, whereas SCID mice mimic the clinical situation in AIDS and other disorders of cell-mediated immunity. Recently, intracerebral administration of chloroquine was shown to increase median survival time and decrease yeast growth in the brains of mice given a lethal intracerebral inoculum of *C. neoformans* (43). Newman et al. (5) also found that chloroquine was therapeutic in a murine model of histoplasmosis, although they used a substantially higher dosage of chloroquine than was used in our studies.

Chloroquine has excellent bioavailability after oral administration, and, at usual therapeutic dosages, serum concentrations of 1–1.5  $\mu$ M are obtained (5, 44). However, chloroquine accumulates in phagocytes, and it has been estimated that cellular levels comparable to those found during antirheumatic therapy are achieved by the in vitro incubation of cells with 100  $\mu$ M chloroquine (45). This concentration is 10-fold greater than the concentration of chloroquine necessary to empower mononuclear phagocytes for enhanced anticryptococcal activity in our experiments. Moreover, chloroquine is greatly concentrated in certain tissues, where mM concentrations can be obtained. Taken together with our findings that chloroquine

was protective in animal models, our data lend support for clinical trials evaluating chloroquine for adjunctive treatment and prophylaxis of cryptococcosis. In addition to its once-weekly oral dosage and low toxicity, chloroquine has a potential advantage over antifungal agents (such as amphotericin B and fluconazole) in that it does not have a direct effect on the fungus. Thus, the likelihood of resistance developing should be relatively small. Moreover, chloroquine may be beneficial for other intracellular pathogens that affect individuals with impaired cell-mediated immunity, as experimental data with several of these pathogens suggest (4–6, 46). However, because chloroquine can interfere with effective antigen-processing (47), the greatest benefit might be in those patients with severely impaired T cell function, particularly individuals with AIDS and CD4+ T cell counts < 100/ $\mu$ l blood. Antigen-processing is probably superfluous in such patients due to their inability to mount a T cell response. Interestingly, the chloroquine analogue hydrochloroquine has been shown to inhibit replication of HIV-1 in infected T cells and monocytes (48). In addition, in a small clinical trial, HIV-infected individuals treated with hydrochloroquine had a significant decrease in their total plasma levels of HIV-1 RNA (49).

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