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Enhanced Resistance to *Cryptococcus neoformans* Infection Induced by Chloroquine in a Murine Model of Meningoencephalitis

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Although the pathogenesis of cerebral cryptococcosis is poorly understood, local immune cells, such as microglia and astrocytes, likely play a critical role in containing infection. Chloroquine (CQ) is a weak base that accumulates within acidic vacuoles and increases their pH. Consequently, proteolytic activity of lysosomal enzymes and intracellular iron release/availability are impaired, resulting in decreased availability of nutrients crucial to microorganism survival and growth in the host. We found that CQ enhances BV2 microglial-cell-mediated anticryptococcal activity in vitro. The phenomenon is (i) evident when both unopsonized and opsonized microorganisms are used and (ii) mimicked by NH_4Cl, another weak base, and by bafilomycin A_1, an inhibitor of vacuolar-type H^+-ATPases. In vivo, intracerebral administration of CQ before lethal local challenge with *Cryptococcus neoformans* results in a significant augmentation of median survival time and a marked reduction of yeast growth in the brain and is associated with the enhancement of local interleukin 1β (IL-1β) and IL-6 mRNA transcripts. Overall, these results provide the first evidence that CQ enhances anticryptococcal host defenses.

Cryptococcus neoformans is a significant cause of opportunistic infections with a marked predilection for the central nervous system (38). Acquired by inhalation, cryptococcosis may occur as an asymptomatic pulmonary infection; however, in immunocompromised hosts, it may result in a fatal disseminated disease generally manifested as meningitis (16).

Numerous in vitro studies indicate that monocytes, macrophages (Mϕ), natural killer cells, and polymorphonuclear cells exhibit fungicidal activity against *C. neoformans* (28, 34, 36). Despite the importance of innate immunity, the capacity of the host to mount an adequate cell-mediated immune response plays an important role in resistance to cryptococcosis. In particular, $CD4^+$ lymphocyte depletion allows rapid dissemination of the fungus from the lung to other organs, including the brain (29, 35).

Chloroquine (CQ) is a drug widely used against malaria and certain kinds of chronic inflammatory diseases (31, 42, 44). Experimentally, it is also used to study the role of vacuole acidification in immune cell functions (30, 39). Indeed, CQ is a weak base that accumulates in acidic vacuoles (endosomes, lysosomes) and increases their pH. Consequently, both the enzymatic activities of such endocellular compartments and the availability of nutrients crucial to microorganism survival and growth are impaired (25, 37, 40).

In previous reports, we demonstrated that brain immune defense mechanisms have a role in the onset and development of cryptococcal meningoencephalitis. In this respect, initial evidence indicates that brain phagocytic effector cells exert local antimicrobial activities (2, 3, 7). In particular, in vitro studies document that microglial Mϕ are proficient anticryptococcal effectors, provided they have been exposed to gamma interferon and/or that opsonized microorganisms are used (3, 5). Moreover, it has been shown that in vivo pharmacological impairment of phagocytic functions is detrimental to the host (2, 3), while intracerebral (i.c.) administration of picolinic acid, a potent Mϕ costimulus (9), or of heat-killed *C. neoformans*, which is known to retain immunostimulating properties (8), enhances host resistance to a subsequent lethal challenge with yeast cells. This event is associated with a local response in terms of enhanced levels of mRNA transcripts specific for interleukin 1β (IL-1β) and IL-6, indicating that brain anticryptococcal defenses involve local production of phagocyte-related cytokines (4).

Using a murine model, we investigated the effects of CQ in vitro on anticryptococcal activity of microglial cells and in vivo on the establishment of cryptococcal meningoencephalitis. We show that in vitro CQ enhances BV2 cell anticryptococcal activity. This phenomenon is evident when unopsonized as well as opsonized microorganisms are used and is mimicked by NH_4Cl, another weak base, and by bafilomycin A_1 (BAF), an inhibitor of the vacuolar-type H^+-ATPases. Moreover, in vivo i.c. administration of CQ before lethal local challenge with *C. neoformans* results in (i) significant augmentation of median survival time (MST), (ii) marked reduction of fungal brain colonization, and (iii) local induction of IL-6 and tumor necrosis factor alpha (TNF-α) gene expression. Overall, these results provide the first evidence that CQ may enhance host resistance to *C. neoformans* i.c. infection.

MATERIALS AND METHODS

Mice. Female C57BL/6 (*H-2*^{b}) mice, 6 to 8 weeks old, 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 agar plates, washed twice in saline by low-speed centrifugation (1,000 × *g*),

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and diluted to the appropriate concentration in RPMI 1640 medium or saline prior to use in the in vitro or in vivo assays.

C. neoformans was opsonized as follows. Microorganisms (10^5) were incubated for 20 min at 37°C with 50 μg of monoclonal antibody 2H1 (immunoglobulin G1), which binds glucuronoxylomannan and was kindly supplied by A. Casadevall (Departments of Medicine and of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, N.Y.), in a total volume of 200 μl of RPMI 1640. Then the yeast cells were washed, and appropriate dilutions for the in vitro assays were prepared in RPMI 1640 complete medium.

Cell lines. The BV2 microglial cell line used in this study was obtained as previously described (1). Briefly, primary microglial cultures were infected with a v-*raf* and v-*myc* oncogene-carrying retrovirus and maintained by biweekly passages in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Hyclone), gentamicin (50 μg/ml), and L-glutamine (2 mM).

Drugs. CQ, NH_4Cl, and BAF were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions, prepared with sterile pyrogen-free saline, were stored at −80°C and used in the experiments described. Only preparations showing lipopolysaccharide contamination of <0.5 ng/ml (as detected by *Limulus* lysate assay) were used.

Measurement of anticryptococcal activity. BV2 microglial cells were plated (5×10^5 cells/ml) in 96-well plates (Corning Glass Works, Corning N.Y.) and incubated with the drugs for 1 h. Then *C. neoformans* (5×10^4/ml) was added. After different hours of incubation at 37°C in 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 to 72 h of incubation at room temperature. Control cultures consisted of *C. neoformans* incubated without effector cells. Results were expressed as percentage of anticryptococcal activity, according to the following formula:

$$\%\ \text{anticryptococcal activity} = 100 - \frac{\text{CFU for experimental groups}}{\text{CFU for control cultures}} \times 100$$

Inoculation. Inoculations (i.c.) were performed on anesthetized mice as detailed below. Mice received inocula (30 μl per mouse) in 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. CQ-treated mice received CQ, and untreated mice received saline according to schedules described below prior to i.c. challenge with *C. neoformans*. Mice recovered from trauma within 30 to 60 min. Surgical mortality was less than 3% and always occurred within 1 to 5 min after inoculation.

Quantitation of *C. neoformans* in the brain. At different times after challenge, brains from individual mice (three per group) were removed aseptically and placed in a tissue homogenizer with 3 ml of sterile distilled water. The number of CFU was determined by a plate dilution method on Sabouraud dextrose agar. Colonies of *C. neoformans* were counted after 48 or 72 h of incubation at room temperature, and the results were expressed as the number of CFU per organ.

RNA extraction. Total RNA was isolated from the brains of six mice per experimental group by solubilization with guanidine isothiocyanate as previously described (15). Following digestion in DNase, a 15-μg aliquot of RNA was electrophoresed on a 1% agarose formaldehyde denaturing gel containing ethidium bromide to detect the intact 18S and 28S rRNAs and to confirm the integrity of the isolated RNA. The amount of RNA was calculated by measuring the optical density at 260 nm in a spectrophotometer (Beckman). Total RNA was then processed for cDNA synthesis and PCR amplification assay.

RT. Following heating at 65°C for 3 min and subsequent chilling on ice, a 5-μg aliquot of total RNA in 13.66 μl of diethylpyrocarbonate-treated water was used in each reverse transcription (RT) reaction. RT buffer for each sample contained the following: 1.1 μl of 1 M Tris-HCl (pH 8.3) (Bethesda Research Laboratories [BRL], Gaithersburg, Md.), 0.13 μl of 1 M $MgCl_2$ (BRL), 0.5 μl of 1 M KCl (BRL), 0.22 μl of 150 mM dithiothreitol (Promega, Madison, Wis.), 1.33 μl of deoxynucleoside triphosphates (25 mM each; Promega), 1.4 μl of oligo$(dT)_{15}$ primer (0.8 μg/μl; Promega), 0.33 μl of RNasin (40 U/μl; Promega), and 1.33 μl of avian myeloblastosis virus reverse transcriptase (7.5 U/μl; Promega). The total reaction volume was 20 μl, and the reaction was allowed to continue at 42°C for 90 min. The reaction was stopped by the addition of 980 μl of TE buffer (Tris-EDTA, pH 6.2) (final volume of each sample of cDNA, 1 ml).

PCR. The PCR mixture for each sample contained 6.5 μl of double-distilled sterile water, 3.2 μl of 10× PCR buffer (Pharmacia, Uppsala, Sweden), 3.2 μl of 1.25 mM deoxynucleoside triphosphates (Promega), 1 μl each of 3′ and 5′ primers (25 pM final concentration; Promega), and 0.1 μl of *Taq* polymerase (5 U/μl; Pharmacia). Each cycle consisted of denaturation at 94°C for 1 min; annealing at 55°C for 2 min (for IL-12p40), 60°C for 1 min (for β-actin, TNF-α and IL-6), or 65°C (for IL-1β and inducible nitric oxide synthase); and extension at 72°C for 1 min. Before each cycle, the samples were heated to 100°C for 2 min and then cooled to 80°C before being added to the reaction mixture. Amplification was repeated for 30 cycles in a Perkin-Elmer Cetus DNA thermal cycler. Ten microliters of the PCR amplification products were separated on an ethidium bromide-stained 1.5% agarose gel, visualized by UV transillumination, and photographed. Aliquots of 0.05 μg of ϕX174 replicative-form DNA-*Hae*III fragments (New England BioLabs, Beverly, Mass.) were run in parallel as molecular size markers (providing bands at 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp). The amplified bands showed their predicted sizes. Cytokine-specific primers were DNA specific and nonreactive with RNA. The following oligonucleotide 5′ and 3′ primer sequences (synthesized in our laboratory) were used: TNF-α, AGCCCACGTCGTAGCAAACCACCAA and ACA CCCATTCCCTTCACAGAGCAAT; IL-6, ATGAAGTTCCTCCTGCAAGA GAC and CACTAGGTTTGCCGAGTAGATCTC; IL-1β, TGAAGGGCTGC TTCCAAACCTTTGACC and TGTCCATTGAGGTGGAGAGCTTTCAGC; iNOS, CCCTTCCGAAGTTTCTGGCAGCAGC and GGCTGTCAGAGCCTC GTGGCTTTGG; IL-12p40, CAGAAGCTAACCATCTCCTGGTTTG and TCCGGAGTAATTTGGTGCTTCACAC; and β-actin, CTGAAGTACCCCAT TGAACATGGC and CAGAGCAGTAATCTCCTTCTGCAT. Positive-control DNAs for each cytokine were obtained from Clontech Laboratories, Palo Alto, Calif., while negative controls consisted of samples in which (i) RNA was replaced by diethylpyrocarbonate plus distilled water, (ii) RT was omitted to detect any contamination by previously amplified cDNA, and (iii) the primers were not added.

Statistical analysis. Differences in survival times were determined by the Mann-Whitney U test. Differences in the number of CFU were determined by Student's *t* test.

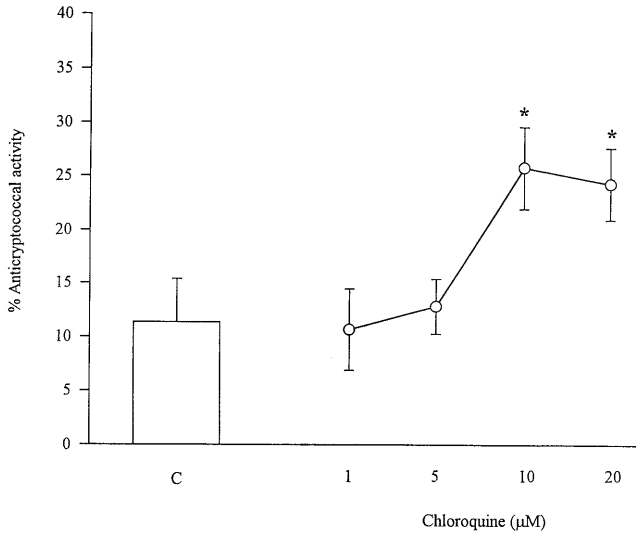

FIG. 1. Effects of different CQ concentrations on anticryptococcal activity by BV2 microglial cells. BV2 microglial cells (5×10^5 cells/ml) were cultured for 1 h in medium alone (c) or in medium containing increasing concentrations of CQ. Then *C. neoformans* (5×10^4 yeast cells/ml) was added, and after 8 h of coincubation, a CFU assay was performed as detailed in Materials and Methods. Data shown are the means ± standard deviations of three independent experiments. *, $P < 0.01$ (CQ-treated versus untreated cells).

RESULTS

To investigate the effects of CQ on BV2 cell anticryptococcal activity, we performed in vitro experiments in which BV2 microglial cells were incubated for 1 h alone or in the presence of CQ at concentrations ranging from 1 to 20 μM. Then BV2 cells were infected with *C. neoformans* at an effector-to-target ratio of 10:1, and after 8 h of coincubation, a CFU assay was performed. As reported in Fig. 1, CQ increased the percentage of CFU inhibition by BV2 cells in a concentration-dependent manner. In particular, maximal levels of such activity were evident at a CQ concentration of 10 μM. As assessed by trypan blue exclusion and lactate dehydrogenase release, CQ was not toxic for BV2 cells. Similarly, the presence of CQ did not alter the viability and growth of *C. neoformans* as evaluated by CFU assay (data not shown).

To better characterize this phenomenon, kinetic studies on BV2 cell anticryptococcal activity were performed. BV2 cells, incubated alone or with CQ (10 μM) for 1 h, were infected with *C. neoformans*. CFU assays were performed at different times. The results obtained by comparing untreated versus CQ-treated BV2 cells are depicted in Fig. 2. While at 2 and 4 h

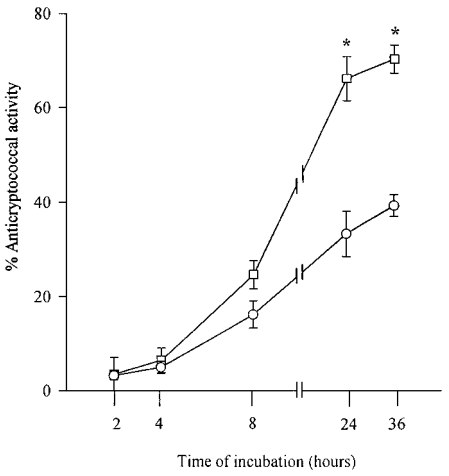

FIG. 2. Kinetics of anticryptococcal activity by BV2 microglial cells treated or not with CQ. BV2 microglial cells (5×10^5/ml) were incubated for 1 h with (□) or without (○) CQ (10 μM). Then *C. neoformans* (5×10^4/ml) was added, and CFU assays were performed. Percentage of anticryptococcal activity was calculated as described in Materials and Methods. Data shown are the means ± standard deviations of three independent experiments. *, $P < 0.01$ (CQ-treated versus untreated cells).

there were no significant differences between the two experimental groups, at 8 h, the percentage of CFU inhibition was significantly augmented in CQ-treated BV2 cells, and maximal differences were observed at later time points (Fig. 2). We showed previously that BV2 cells exhibit enhanced anticryptococcal activity when opsonized yeast cells are used (3). In order to establish whether CQ effects might be further enhanced, BV2 cells were treated with CQ (10 μM) for 1 h and then exposed to opsonized *C. neoformans*. After 8 and 24 h, a CFU assay was performed. Unopsonized yeast cells were included as baseline controls. When opsonized yeast cells were used, anticryptococcal activity of CQ-treated BV2 cells was consistently higher than that of untreated BV2 cells: 75 versus 42% and 96 versus 62% at 8 and 24 h, respectively (Table 1). As expected, CQ pretreatment enhanced BV2 cell anticryptococcal activity

TABLE 1. Effect of lysosomotropic compounds on BV2 microglial cell anticryptococcal activity

Lysosomotropic compound[a]	Concn	Anticryptococcal activity against *C. neoformans*[b]			
		Unopsonized		Opsonized	
		8 h	24 h	8 h	24 h
None		14	34	42	62
CQ	10 μM	25[c]	66[c]	75[c]	96[c]
NH_4Cl	20 mM	28[c]	73[c]	56[c]	93[c]
	10 mM	26[c]	62[c]	40	88[c]
	5 mM	10	54[c]	37	87[c]
BAF	300 nM	20	60[c]	60[c]	70[c]
	200 nM	15	30	40	58
	150 nM	10	31	35	50

[a] BV2 cells were treated with CQ (10 μM) or different concentrations of NH_4Cl or BAF for 1 h. Then *C. neoformans* was added to the cultures.
[b] Anticryptococcal activity was assessed at 8 and 24 h against unopsonized or opsonized *C. neoformans* as detailed in Materials and Methods. Data shown are the means of three independent experiments. Standard deviations, which were less than 5%, have been omitted.
[c] $P < 0.01$ (treated BV2 cells versus untreated).

TABLE 2. Effect of CQ on survival of mice challenged i.c. with *C. neoformans*

Treatment with CQ (μM)[a]	*C. neoformans* i.c. challenge[b]	MST (days)	Survival range (days)	D/T[c]
None	10^4	20	10–30	30/30
10	10^4	30[d]	15–45	30/30
1	10^4	48[e]	23–60	30/30
0.1	10^4	22	11–28	30/30

[a] Mice received CQ or saline i.c. 24 and 3 h before challenge.
[b] *C. neoformans* (10^4 cells per mouse) was given i.c. on day 0.
[c] Number of dead mice at 60 days/total number of animals tested.
[d] $P < 0.05$ (treated versus untreated mice).
[e] $P < 0.01$ (treated versus untreated mice).

against unopsonized microorganisms at both time points (Table 1).

Using parallel groups, we examined the effects of two other lysosomotropic compounds, NH_4Cl (a weak base) and BAF (an inhibitor of vacuolar-type H^+-ATPases), on the anticryptococcal activities of BV2 cells. For this purpose, BV2 cells were treated with different concentrations of NH_4Cl or BAF for 1 h and then assessed for activity against *C. neoformans* 8 and 24 h later (Table 1). Both NH_4Cl and BAF increased the anticryptococcal activities of BV2 cells. The effects were concentration dependent, observed after 8 and 24 h of infection, and evident against both unopsonized and opsonized yeast cells (Table 1). When BV2 cells were assessed for phagocytosis, we found no differences between untreated BV2 cells and CQ-, NH_4Cl-, or BAF-treated BV2 cells, regardless of whether unopsonized or opsonized targets were used (data not shown).

To test the possibility that CQ might be effective in vivo, we performed dose-response experiments in which mice were injected with different doses of CQ prior to local lethal challenge with *C. neoformans*. In particular, CQ at different doses or placebo were inoculated i.c. both 24 and 3 h before challenge (10^4 live yeast cells/mouse). As depicted in Table 2, a significant enhancement of MST was observed in CQ-treated versus placebo-treated mice (48 versus 20 days). An optimal effect was observed at a CQ concentration of 1 μM, while a concentration of 10 μM was less effective and 0.1 μM was totally ineffective (Table 2). On these bases, we evaluated brain colonization in CQ- and placebo-treated mice after *C. neoformans* challenge by CFU assay (Table 3). Mice treated with CQ (1 or 10 μM) showed a significant reduction in brain CFU from 1 to 10 or 1 to 3 days postinfection, respectively. In contrast, treatment with a low CQ concentration (0.1 μM) had no effect on yeast cell colonization of the brain.

To establish whether CQ-mediated effects could be associ-

TABLE 3. *C. neoformans* recovered from brains of CQ-treated and untreated mice

Treatment with CQ (μM)[a]	CFU (10^3) of *C. neoformans* recovered from brains[b]			
	Day 1	Day 3	Day 7	Day 10
None	1.4	83.9	1,359	3,258
10	0.7[c]	63.2[c]	1,023	3,635
1	0.2[c]	44.6[c]	741[c]	1,055[c]
0.1	1.0	86.5	1,005	3,483

[a] Mice were treated i.c. with CQ or saline 24 and 3 h before i.c. challenge with *C. neoformans* (10^4 cells per mouse).
[b] CFU recovery from brains was assessed at the indicated days as detailed in Materials and Methods. Data shown are the means of three independent experiments. Standard deviations, which were less than 5%, have been omitted.
[c] $P < 0.01$ (treated versus untreated mice).

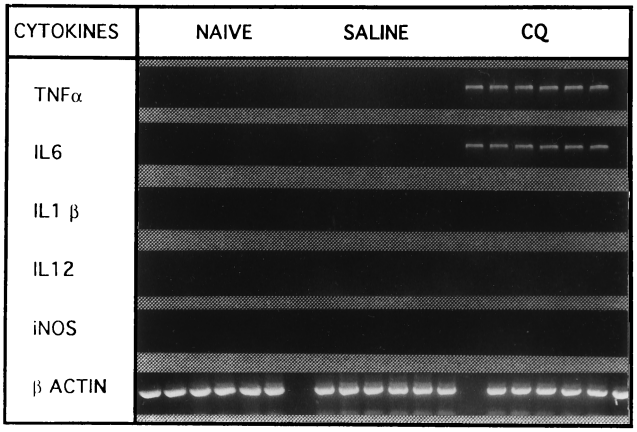

FIG. 3. Effect of CQ i.c. administration on brain cytokine gene expression. Mice were treated i.c. with saline or CQ (1 μM) 24 and 3 h earlier. At time 0 before *C. neoformans* challenge, the animals were sacrificed. Brains were harvested and processed, and RNA was employed for RT-PCR analysis as detailed in Materials and Methods. Results from six brains per experimental group are shown.

ated with the modulation of local cytokine gene expression, molecular studies were performed by RT-PCR analysis. For this purpose, untreated naive mice and saline- or CQ-treated (−24 and −3 h) mice were sacrificed at time 0 before *C. neoformans* challenge. Brains were isolated and processed for assessment of TNF-α-, IL-6-, IL-1β-, IL-12-, and iNOS-specific mRNA levels. As depicted in Fig. 3, naive and saline-treated mice showed undetectable levels of all gene transcripts investigated. In contrast, CQ treatment resulted in the expression of TNF-α and IL-6 genes in six of six mice assessed, while IL-1, IL-12, and iNOS gene transcripts remained undetectable. β-Actin transcripts were detected in all the samples tested.

DISCUSSION

During inflammatory processes and/or infections in the brain, local defense elements, such as microglia and astrocytes, have several immunological functions, including phagocytosis (1, 43), antigen presentation (21, 22, 24), cytokine production (20, 23, 26, 27, 33), respiratory burst, and antimicrobial activities (14, 32, 45). In particular, microglial cells are proficient anticryptococcal elements in vitro. Our previous studies demonstrated that BV2 microglial cells are capable of ingesting *C. neoformans* and inhibiting its growth. This is accomplished in a time- and effector-to-target-ratio-dependent fashion and is markedly potentiated upon opsonization of the target (3). In the present report, we demonstrate that, following in vitro exposure to CQ, BV2 microglial cells exhibit increased anticryptococcal activity, which is particularly evident at a CQ concentration of 10 μM and after 8 and 24 h of infection with *C. neoformans*. This finding is in line with several reports demonstrating that murine as well as human Mϕ are susceptible to CQ and respond with enhanced antimicrobial properties (12, 18, 37, 41). The molecular mechanisms involved in this phenomenon have been partially identified. Indeed, CQ interferes with the endosomal/lysosomal functions. By raising intravacuolar pH, CQ inhibits (i) the proteolytic activities of lysosomal enzymes (40) and (ii) the release of iron from its transferrin binding sites within the endosome (12, 42). In our experimental system, we observed enhancement of anticryptococcal activity in BV2 cells exposed to CQ as well as to other compounds, such as NH_4Cl or BAF, which are known to impair intracellular acidification processes through similar (NH_4Cl) or different (BAF) mechanisms (11). Altogether, our results strongly suggest that within certain subcellular compartments, pH is important for the accomplishment of BV2 cell anticryptococcal activity. It remains to be elucidated whether CQ acts on BV2 cells via alteration of proteases and/or of iron metabolism. We tend to exclude the second hypothesis. In a recent report, we established that anticandidal activity of BV2 cells is potentiated by iron loading (via iron nitrilotriacetate [FeNTA]). Thus, it appears that two compounds, CQ and FeNTA, which are known to mediate opposite effects in terms of intracellular iron availability, both enhance antifungal activity. The mechanisms through which CQ acts on BV2 cells are therefore probably related to alteration of lysosomal enzymatic activities rather than to interference with iron metabolism. This conclusion is supported by preliminary data showing that BV2 cell exposure to FeNTA does not abrogate the CQ anticryptococcal effect (data not shown).

According to the literature, enhancement of intravacuolar pH can be detrimental to some intracellular pathogens, probably causing a reduction in the availability of nutrients critical for their survival and growth via inactivation of the acidic proteases (11, 25). On these bases, we can speculate that, once ingested by CQ-treated cells, *C. neoformans* encounters an intracellular environment that is less favorable, as it is deprived of crucial nutrients. This hypothesis is supported by the fact that promotion of phagocytosis via *C. neoformans* opsonization is associated with the expression of maximal levels of anticryptococcal activity by CQ-treated BV-2 cells. Finally, the induction of autocrine activation loops by CQ is unlikely, since we failed to detect TNF-α, IL-6, IL-12, and nitric oxide in supernatants of BV2 cells exposed to CQ and then infected with *C. neoformans* (data not shown).

We have previously described an experimental model of i.c. infection with *C. neoformans* in which mice develop a lethal disease associated with a massive colonization of the brain (3). Here, we show that CQ significantly reduces host susceptibility to lethal i.c. infection with *C. neoformans*. Our data demonstrate that injection of this compound directly into the brain results in a twofold increase in the MST of lethally infected mice. This phenomenon, which is accompanied by a marked delay in fungal colonization of the brain, is most evident at the 1 μM concentration, while at 10 μM, the benefit of CQ is only partial. Whether toxic effects or rather down-regulating feedback signals may be involved is currently being investigated. Since the efficacy of CQ against malaria and histoplasmosis has been documented (31, 37), these results include cryptococcosis as another infectious disease against which CQ may be beneficial.

Evidence indicates that immunocompetent brain elements, such as microglia and astrocytes, have many functions during inflammatory and/or infectious diseases at the cerebral level. In particular, they exhibit phagocytic activity, act as potent antigen-presenting cells, and produce and are stimulated by numerous biological products, including cytokines, whose autocrine and paracrine roles as enhancers of antimicrobial functions have been described elsewhere (13). Using RT-PCR analysis, we demonstrate here that, unlike control brains from naive or saline-treated mice, brains from CQ-treated mice show induction of TNF-α and IL-6 gene expression. The phenomenon is highly consistent (six of six mice) and does not involve all cytokine genes, since IL-1β, IL-12, and iNOS gene transcripts remain undetectable. To our knowledge, this is the first evidence ascribing an immunostimulating role to CQ. In this respect, it is of interest that a closely related compound, hydroxychloroquine, has recently been reported to improve

several immunological parameters in human immunodeficiency virus-infected patients (41). In contrast, numerous reports document the wide spectrum of anti-inflammatory properties of CQ in vivo. Because of them, CQ is given as a useful therapeutic agent in several forms of arthritis and in experimental models of hemorrhagic shock, where monocyte/macrophage secretory functions appear to be CQ inhibited (17, 19). While further studies are required to better elucidate the phenomenon, our results imply that the brain compartment behaves differently from other tissues/organs, thus elaborating a peculiar biomolecular response(s) to a chemical compound such as CQ. In this respect, it is worth noting that once stimulated, Mϕ of diverse anatomical origins express different patterns of effector and secretory functions (10). Also, the existence of a cell type other than the Mϕ that is specific to the brain and is capable of elaborating a positive response to CQ (IL-6 and TNF gene expressions) is conceivable. Experiments will be performed to establish the susceptibility of astrocytes and endothelial or neuronal cells to CQ in vitro. Regardless of the responder cell type, CQ treatment is beneficial to the host in our experimental model. Interestingly, the phenomenon occurs only if the compound is given i.c. Administration of CQ intraperitoneally fails to affect mouse survival after cryptococcal i.c. challenge (data not shown), further strengthening the possibility of involvement of cerebral defense elements in the above-described phenomenon. As previously shown (4), the periphery is little or not at all related to the establishment of brain resistance. We consistently found induction of brain cytokine-specific gene expression in mice rendered resistant to lethal i.c. cryptococcal challenge by appropriate i.c. manipulation (4, 6).

In conclusion, while the exact mechanism(s) by which CQ confers brain anticryptococcal resistance remains to be clarified, we can speculate that there is at least a dual effect on brain immune cells: (i) enhancement of pH within the endosomal/lysosomal compartment and (ii) induction of cytokine gene expression. By acting separately and/or synergistically, such mechanisms may contribute to the observed containment of cerebral cryptococcosis in vivo.

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