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Biomolecular Events Involved in Anticryptococcal Resistance in the Brain

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We have recently shown that intracerebral (i.c.) administration of heat-killed *Cryptococcus neoformans* (HCN) enhances mouse resistance to a subsequent local challenge with lethal doses of viable yeast cells. Here we show that i.c. administration of HCN is also effective in significantly delaying brain colonization of mice intravenously infected with viable *C. neoformans*. PCR analysis revealed that interleukin 6 (IL-6) and IL-1β gene expression occurs in brains of HCN-treated mice but not in brains of saline-treated controls. In contrast, no differences are observed in terms of tumor necrosis factor alpha and IL-1α gene transcripts, which are slightly and highly detectable, respectively, in saline-treated mice and which remain such also following HCN treatment. Furthermore, i.c. administration of exogenous IL-6 or IL-1β, but not tumor necrosis factor alpha, before local challenge with viable *C. neoformans* results in significantly reduced microbial counts in the brain and blood and in increased mouse survival. Taken together, these observations provide initial evidence that brain anticryptococcal resistance involves elicitation of a local cytokine response, involving primarily IL-6 and IL-1β.

Life-threatening disease caused by *Cryptococcus neoformans* has increased significantly as a result of AIDS and immunosuppressive drug therapies (13, 18). Cryptococcosis may occur as a pulmonary infection, which in the immunocompromised host can evolve to a fatal disseminated disease, generally manifested as meningitis (37). In spite of aggressive chemotherapy, patient relapse and mortality rates are high. In this respect, a major goal is the development of successful immunotherapeutic protocols that, nevertheless, cannot be accomplished without a comprehensive understanding of the anticryptococcal host defenses, including effector cells and cytokines involved in the resistance against this fungal pathogen.

Evidence exists that monocytes and macrophages (Mϕ), natural killer cells, and polymorphonuclear cells exhibit fungicidal activity against *C. neoformans* in vitro (16, 17, 24, 25, 27, 28, 30, 32, 33, 36, 39). Cytokines such as tumor necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor also appear to be involved in anticryptococcal defenses, being known to enhance complement-dependent *C. neoformans* phagocytosis by murine Mϕ (14). Furthermore, acquired cell-mediated immunity plays an important role in host defense against cryptococcosis (9, 10, 26, 30, 35). In particular, $CD4^+$ lymphocyte depletion allows rapid dissemination of the fungus from the lung to other organs, including the brain (34).

To date, scant information exists on the defense mechanisms evoked locally during cerebral cryptococcosis. Initial evidence indicates that brain phagocytic effector cells exert local antimicrobial responses (5, 31). In particular, in vitro studies document that microglial Mϕ are proficient anticryptococcal effectors provided that opsonized microorganisms are used (3). In addition, in vivo murine models have been used to show that pharmacological impairment of phagocytic functions is detrimental to the host (2). Conversely, intracerebral (i.c.) administration of a potent Mϕ costimulus (4, 7) or heat-killed *C. neoformans* (HCN) (6) enhances host resistance to lethal i.c. challenge with the fungus. In particular, HCN i.c. injection allows a local immune reaction to the subsequent challenge, which, in turn, results in impairment of its pathogenic potential (6).

The aim of our study was to evaluate whether brain antifungal resistance, induced by HCN i.c. administration, occurred through mechanisms likely involving local production of phagocyte-related cytokines. Thus, by using PCR amplification methods, we demonstrated that HCN treatment, per se, induces interleukin 1β (IL-1β) and IL-6 gene expression in the mouse brain, while no changes occur in terms of TNF alpha (TNF-α) and IL-1α gene transcripts. Moreover, i.c. administration of exogenous IL-1β and IL-6, but not TNF-α, significantly enhances host resistance, further strengthening the conclusion that a role is played by certain cytokines as potent immunomodulators against cerebral cryptococcosis.

MATERIALS AND METHODS

Mice. C57BL/6 ($H\text{-}2^b$) 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. The yeast was maintained by biweekly passages on Sabouraud dextrose agar plates and 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 pyrogen-free saline prior to use for systemic (intravenous [i.v.]) or i.c. inoculations. HCN was obtained by heating the microorganisms three times at 121°C. Stock solutions were tested for endotoxin contamination by the *Limulus* amebocyte lysate assay. Only preparations with undetectable endotoxin levels (less than 0.5 ng/ml) were kept at −20°C until used.

i.c. inoculation. i.c. inoculations of anesthetized mice were performed as described previously (38). Briefly, yeast cells were suspended in pyrogen-free saline and inoculated (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.

Recombinant cytokines. Recombinant human IL-6 (Boehringer Mannheim

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Biochemicals, Mannheim, Germany), recombinant human IL-1β, and TNF-α (Genzyme Corp., Cambridge, Mass.) were diluted in sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and stored at −80°C until used. Treated mice received cytokines (1,000 U per mouse) at 24 and 3 h before microbial challenge, and untreated (or saline-treated) mice received PBS plus 0.1% BSA (placebo) at the same times.

Quantitation of *C. neoformans* in organs. Brains or spleens of mice were removed aseptically and placed in a tissue homogenizer with 3 ml of sterile distilled water. Blood samples (100 μl per mouse) were obtained by retro-orbital sinus bleeding before the mice were killed. The number of CFU in the specimens was determined by a plate dilution method on Sabouraud dextrose agar. Colonies of *C. neoformans* were counted after 48 and 72 h of incubation at room temperature, and the results were expressed as the number of CFU per organ or number of CFU per milliliter of blood.

RNA extraction. Total RNA was isolated from the brains or spleens of three mice per experimental group by solubilization with guanidine isothiocyanate by the method of Chomczynski and Sacchi (12). 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 rRNA 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). Three aliquots of total RNA were made from each sample and processed separately for cDNA synthesis and a PCR amplification assay.

Reverse transcription. 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 reaction. Reverse transcription 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 (dNTPs; 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 1.5 h. The reaction was stopped by the addition of 980 μl of TE buffer (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 dNTP (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 60°C (for β-actin and TNF-α), 49°C (for IL-6), 59°C (for IL-1α), or 65°C (for IL-1β) for 1 min, 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 was 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 (Promega) were used: TNF-α, GCGACGTGGAACTGGCAGAAG and GGTACAACCCATCGGC TGGCA; IL-6, GTGACAACCACGGCCTTCCCTACT and GGTAGCTATG GTACTCCA; IL-1α, CTCTAGAGCACCATGCTACAGAC and TGGAATC CAGGGGAAACACTG; IL-1β, TGAAGGGCTGCTTCCAAACCTTTGACC and TGTCCATTGAGGTGGAGAGCTTTCAGC; and β-actin, CTGAAGTA CCCCATTGAACATGGC 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) the reverse transcription was omitted to detect any contamination by previously amplified cDNA, and (iii) the primers were not added.

Radioactive hybridization of PCR products. To verify PCR results, PCR products were electrophoresed and transferred to Hyband-N nylon membranes (Amersham International, Amersham, United Kingdom). Oligonucleotides complementary to sequences within the region flanked by the PCR amplification primers were labeled at the 5′ end with T4 polynucleotide kinase (Boehringer Mannheim Biochemicals) and [γ-^{32}P]ATP (7,000 Ci/mmol; Amersham) for use as radioactive probes. Blots were hybridized with probe for 18 h at 45°C, washed for 30 min at room temperature and for 30 min at 45°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and then exposed to X-ray films.

Statistical analysis. Differences were determined by the Mann-Whitney U test. The results reported in the tables and figures are the means ± standard deviations of three experiments. Each group consisted of 10 mice.

RESULTS

We have previously shown that i.c. administration of HCN results in a consistent enhancement of mouse survival in response to subsequent lethal challenge with viable *C. neoformans*. The phenomenon is associated with reduced microbial counts in the brain and a massive local immune response (6). To better understand the events involved in such resistance, molecular studies were performed by PCR analysis of brains or spleens from HCN- and saline-treated mice that were sacrificed on day 0 before challenge. Organs were removed and processed for assessment of cytokine (IL-6, IL-1α, IL-1β, and TNF-α)-specific transcript levels. When the brain was examined, we found that IL-6 and IL-1β mRNA levels were induced in HCN-treated mice compared with that in saline-treated controls, while TNF-α and IL-1α genes were unaffected following HCN treatment (Fig. 1). Under comparable conditions, β-actin mRNA levels remained unchanged in both animal groups (Fig. 1). When the spleen was examined, we found no substantial differences in the levels of cytokine gene transcripts between HCN- and saline-treated mice under the experimental conditions employed (Fig. 1).

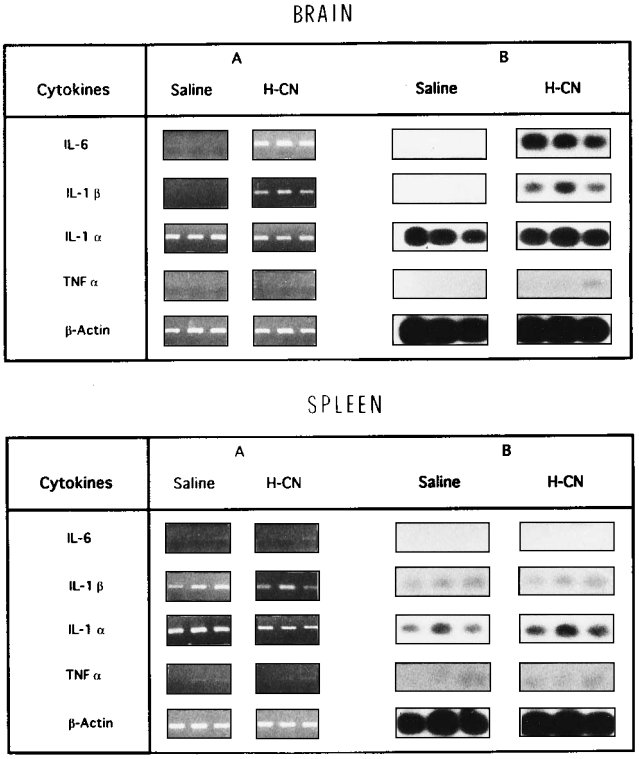

FIG. 1. Detection of cytokine mRNA transcripts by PCR analysis. Three mice per group were treated i.c. on days −14 and −3 with either saline or HCN. On day 0, mice were sacrificed, and brains and spleens were processed for PCR analysis. PCR products from brains (top) and spleens (bottom) were ethidium bromide stained and photographed (A) or blotted and probed with appropriate radioactive oligonucleotides (B) as detailed in Materials and Methods. One representative experiment of two is shown.

To establish the direct involvement of endogenously produced cytokines in local antimicrobial resistance, exogenous IL-6 and IL-1β were administered i.c. (−24 and −3 h) prior to local challenge with 10^4 yeast cells per mouse. As shown in Table 1, mean survival times (MST) were significantly enhanced in cytokine-treated mice compared with that in saline-treated controls. In particular, the extent of the phenomenon was more pronounced in IL-1β- than in IL-6-treated animals (MST = 52 and 40 days, respectively), while control mice died with an MST of 21 days. When as a negative control exogenous TNF-α was administered, we found no significant differences

TABLE 1. Effect of exogenous cytokine administration on survival of mice challenged i.c. with *C. neoformans*

Cytokine administration[a]	Survival after challenge with *C. neoformans*[b]		D/T[e]
	MST[c]	Range[d]	
None	21 ± 1.8	(10–35)	32/32 (100)
IL-1β	52 ± 2.0*[f]	(32–>60)	28/32 (87)
IL-6	40 ± 1.2*	(26–42)	32/32 (100)
TNF-α	26 ± 2.3	(14–37)	32/32 (100)

[a] Mice received IL-1β or IL-6 or TNF-α (10^3 U per mouse; twice) at 24 and 3 h before i.c. challenge with *C. neoformans.*
[b] *C. neoformans* (10^4 yeast cells per mouse) was given i.c. on day 0.
[c] Data are given as MST (days) ± standard deviations.
[d] Survival range (days).
[e] D/T, number of dead mice per total number of animals tested. Percent mortality is shown in parentheses.
[f] *, $P < 0.01$ (cytokine-treated versus saline-treated mice).

in mouse survival: the MST of TNF-α-treated and untreated animals were 26 and 21 days, respectively.

In parallel groups, we evaluated the effect of cytokine treatment on fungal clearance from the brain and blood. Thus, experiments were performed in which mice were sacrificed at various times and organ load was evaluated by CFU assay. Both IL-6 and IL-1β administration resulted in a significant decrease of microbial counts in the brain (Fig. 2A) and blood (Fig. 2B) in cytokine-treated mice compared with that in saline-treated controls, except at 1 h postchallenge, when IL-6 had little or no effect in both the brain and blood. Furthermore, at 24 h, the microbial load in brains of IL-6- and IL-1β-treated mice was still significantly less than that observed in controls, while clearance occurred in the bloodstream in all of the experimental groups. On the basis of survival data, TNF-α administration was almost ineffective in reducing brain and blood microbial counts, showing a significant decrease only at 6 h after *C. neoformans* infection.

In the next series of experiments, HCN- and saline-treated mice were evaluated for their susceptibility to i.v. challenge with *C. neoformans*. We found that HCN-treated and control mice exhibited comparable trends of survival following i.v. challenge (Fig. 3A), while, as expected (6), HCN treatment significantly enhanced host resistance to the i.c. challenge with viable microorganisms (Fig. 3B). In parallel groups, a kinetic CFU assay was performed to assess the microbial counts in the brains and spleens of HCN-treated or untreated mice following i.v. infection. As depicted in Table 2, we found reduced brain colonization in HCN-treated mice compared with that of saline-treated mice, while no differences were observed in terms of microbial load per spleen in the two groups of mice.

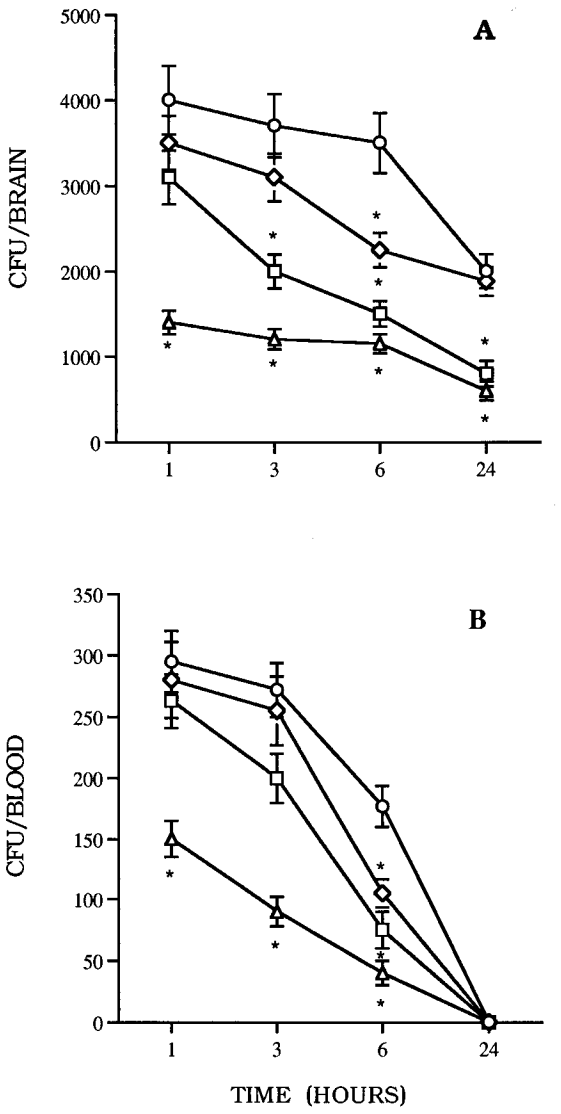

FIG. 2. Kinetics of CFU recovery from the brain and blood of cytokine-treated or saline-treated mice following i.c. challenge with viable *C. neoformans*. Mice were treated i.c. with 10^3 U of IL-6 (□), IL-1β (△), or TNF-α (◇) per mouse or with placebo (○) at 24 and 3 h before challenge. Following challenge (day 0; 10^4 yeast cells per mouse), three animals per group were sacrificed at the indicated times and CFU assays of the brain (A) or blood (B) were done as detailed in Materials and Methods. Bars represent standard deviations. *, significantly different from controls (P of <0.01).

DISCUSSION

We have recently demonstrated that i.c. administration of HCN enhances local antifungal resistance (6). Here we show that this phenomenon is (i) associated with the local induction of cytokine gene expression, (ii) mimicked by i.c. administration of exogenous cytokines, and (iii) related to a delay in brain fungal colonization following i.v. challenge.

Increasing evidence exists indicating that immunocompetent brain elements, such as microglia or astrocytes, exert many functions, including phagocytosis (1), antigen presentation (21, 40), cytokine production (19, 20, 22, 23), and antimicrobial and respiratory burst activities (11, 15, 29, 41) during the inflammatory process and/or infections at the cerebral level. Particularly, cytokines such as IL-1α, IL-1β, IL-6, and TNF-α are known to be produced locally and likely play a crucial role in such events (4, 8). Using PCR analysis, we show here that HCN treatment results in the induction of IL-1β and IL-6 gene expression. Conversely, under the same experimental conditions, TNF-α gene transcripts, undetectable in naive mice (4) (data not shown), remained undetectable regardless of the fact that either saline or HCN had been administered. Similarly, the IL-1α gene constitutively expressed in naive mice (data not shown), remained unaffected. The potential involvement of minimal amounts of contaminating endotoxins in the phenomenon described above has been ruled out by experiments showing that in vitro pretreatment of HCN with polymyxin B does not alter its immunostimulating properties (data not shown).

In a previous report, we established that i.c. administration of picolinic acid (PLA), a potent Mϕ costimulus, protects mice

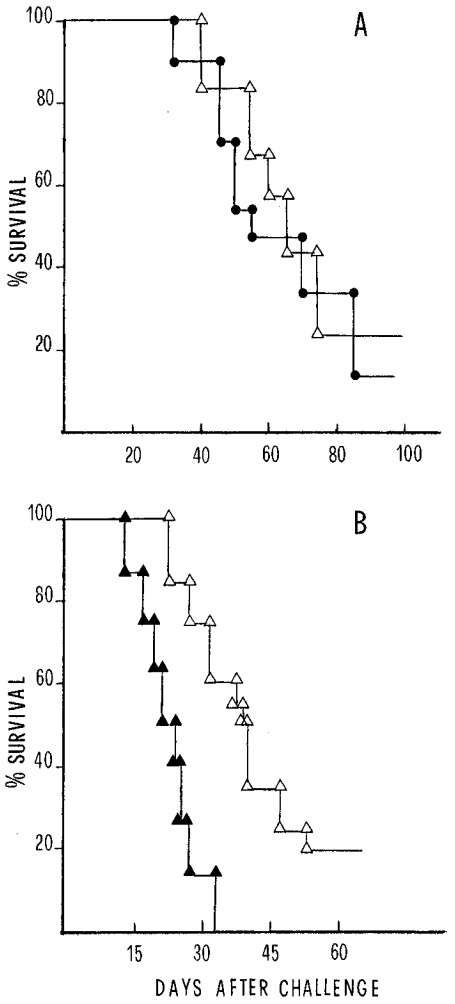

FIG. 3. Survival of HCN- or saline-treated mice after i.v. or i.c. challenge with *C. neoformans*. Mice received HCN (△) (10^7 yeast cells per mouse) or saline (● and ▲) i.c. at 14 and 3 days before challenge. HCN- or saline-treated mice were then challenged i.v. (A) or i.c. (B) with *C. neoformans* (10^4 yeast cells per mouse) on day 0.

TABLE 2. Kinetics of CFU recovery from brains and spleens of HCN-treated mice challenged i.v. with *C. neoformans*

HCN treatment[a]	CFU (10^3) recovered[b]							
	Brain				Spleen			
	+1	+3	+7	+14	+1	+3	+7	+14
No	0.6	29	115	760	0.3	2.3	1.5	0.7
Yes	0.2*[c]	15*	60*	525	0.2	1.0	1.0	0.5

[a] Mice were injected i.c. with 10^7 HCN yeast cells or saline on days −14 and −3.

[b] Mice were challenged i.v. on day 0 (10^4 *C. neoformans* yeast cells per mouse), and CFU assays were performed at the indicated times (days) after infection. Standard deviations were less than 10% and have been omitted for clarity of presentation.

[c] *, $P < 0.01$ (HCN-treated versus saline-treated mice).

against subsequent lethal infection with *Candida albicans* (7). In that model, the resistance was associated with the induction of various cytokine genes, including IL-1β, IL-6, and TNF-α (4). In contrast, we show here that HCN injection induces expression of IL-1β and IL-6 but not TNF-α. Moreover, while the optimal schedule of PLA administration is at 24 and 3 h before microbial challenge (7), HCN is effective only when given several days prior to lethal infection (6). It is conceivable that PLA, a chemical compound, acts directly and/or rapidly on brain-immune cells, thus inducing major changes in local immunity within a few hours; in contrast, HCN requires a multistep and/or time-consuming cascade of immunological events to affect brain antifungal resistance. In any case, these findings indicate that the brain compartment is susceptible to immune stimulation, although to a different extent and through different kinetics, depending upon the inducing agent employed. No matter whether a chemical compound, such as PLA, or a microbial preparation, such as HCN, is administered, the brain immune response involves activation of cytokine genes which affect mainly the phagocytic compartment. In fact, we consistently failed to detect gamma interferon-specific transcripts in brains from HCN- or PLA-treated mice (data not shown). Furthermore, the phenomenon is confined to the brain compartment since cytokine gene activation was not observed in the spleen. Thus, we favor the hypothesis that glial cells are the major HCN responder cells, which, once activated, mediate the local antifungal defenses. This speculation is further supported by initial evidence on the existence of brain anticryptococcal immunity (26), also involving cytokine-activated circuits (29). Nevertheless, in ours as well as other models, the recruitment of inflammatory cells within the brain is a possibility that remains to be established.

Administration of exogenous cytokines prior to lethal challenge with live *C. neoformans* provides direct insight into their involvement in brain antifungal resistance. We show, in fact, that IL-6- or IL-1β-treated mice are less susceptible than controls to i.c. infection in terms of both increased survival and reduced brain microbial load. The beneficial effects of such treatments are evident mainly in the early stages of infection. While control mice need 24 h to significantly reduce the microbial load in the brain, cytokine-treated mice accomplish pathogen clearance to a similar extent within 1 to 6 h. Differences between control and cytokine-treated mice are also observed in terms of microbial counts in the bloodstream, suggesting that the reduced fungal load in the brain of cytokine-treated mice may be ascribed to cerebral effector cells hastened in their ability to locally eliminate the pathogen, thus decreasing its spreading to the periphery. Unlike IL-6 and IL-1β, TNF-α i.c. administration is not beneficial to the host, implying that TNF-α per se has no immunopotentiating properties within the brain. Although far from elucidating the cell types and/or cell functions involved, our results provide the first evidence that (i) brain anticryptococcal resistance can be enhanced provided that the proper cytokine has been employed and (ii) IL-1β and IL-6, but not TNF-α, play a considerable role in enhancing brain anticryptococcal resistance.

The fact that HCN treatment is able to trigger immunological processes resulting in enhanced brain resistance is further supported by experiments showing that i.c. HCN treatment delays brain colonization in systemically infected mice. Nevertheless, such mice eventually succumb to challenge. These results together with the previous demonstration that HCN treatment protects mice (100% survival) against a subsequent lethal challenge with *C. albicans* or *Staphylococcus aureus* (6) further emphasize the beneficial effects of HCN as an enhancer of brain antifungal resistance and underline the resistance of *C. neoformans* to both naive and potentiated immune defenses.

Overall, we conclude that HCN administration induces IL-1β and IL-6 gene expression. While formal proof of cyto-

kine production or release within the brains of HCN-treated mice has yet to be given, we can speculate that local activation processes occur through a cascade of autocrine and paracrine regulatory signals, which, in turn, enhance brain anticryptococcal defenses.

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