Effect of Macrophage Colony-Stimulating Factor on Anticryptococcal Activity of Bronchoalveolar Macrophages: Synergy with Fluconazole for Killing

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The antifungal activity of murine bronchoalveolar macrophages (BAM) and their synergy with fluconazole (FCZ) was studied. BAM cultured with tissue culture medium for 48 to 72 h were fungicidal (24 to 39%) in a 3-h killing assay. However, net killing of Cryptococcus neoformans did not continue when culture time was extended to 24 h, although BAM were fungicidal (88 to 98%). Treatment with macrophage colony-stimulating factor (M-CSF; 5,000 U/ml, 48 h) did not significantly increase BAM killing of a low challenge dose in 3-h assays compared with control BAM. However, M-CSF-treated BAM were significantly more fungicidal against higher challenge doses in the 3-h assays. FCZ was not fungicidal at 5 μg/ml but was highly fungicidal (98 and 99% at 24 and 48 h, respectively). M-CSF-treated BAM acted synergistically with FCZ (2.5 μg/ml) for significantly greater killing than control BAM, 55% versus 22% and 90% versus 45% at 24 h and 48 h, respectively. Killing by M-CSF BAM and FCZ (5.0 μg/ml) was significantly (P < 0.01) greater than that by control BAM and FCZ at 48 h. These findings indicate an important collaborative role for BAM and FCZ in killing C. neoformans, and this is enhanced by M-CSF.

Cryptococcus neoformans is an opportunistic fungal pathogen causing serious systemic infections in AIDS, leukemia, and cancer patients undergoing chemotherapy (10, 12). Although neutrophils and monocytes kill a significant percentage of antibody- and/or serum-opsonized yeast cells in short-term in vitro assays (11, 17), human peritoneal and alveolar macrophages are only fungicidal against ingested yeast cells (8, 15, 24). This suggests that yeast cells ingested by tissue macrophages may be protected from fungicidal activity of neutrophils and monocytes and that migration of infected macrophages may account for dissemination to various organs.

Recently, the effects of cytokines on macrophage interaction with C. neoformans have been studied (9). Gamma interferon has been shown to activate murine peritoneal macrophages for enhanced fungicidal activity against C. neoformans (19).

Macrophage colony-stimulating factor (M-CSF) is a 45- to 75-kDa hematopoietic growth factor with specific receptors on monocytes and macrophages and is produced by macrophages, fibroblasts, and vascular endothelial cells (2). M-CSF is present in normal serum (100 to 300 U/ml) and increases during infection (3, 20). M-CSF enhances antibody-dependent cell-mediated cytotoxicity of monocytes and macrophages in vitro (18). Moreover, M-CSF is necessary for in vitro survival, proliferation, and differentiation of murine alveolar macrophages (1, 16).

Although C. neoformans infects by the pulmonary route, interaction of yeast cells with bronchoalveolar macrophages (BAM) from healthy (8) or immunocompromised patients has received little or no attention. Fluconazole (FCZ) is an antifungal agent with desirable pharmacokinetics (23) and has been used as suppressive therapy for cryptococcosis in AIDS patients (22). Here, we report the effect of M-CSF on BAM antifungal activity and synergy with FCZ for killing C. neoformans.

MATERIALS AND METHODS

Mice. Young adult male mice, 8 to 12 weeks old, were used as sources of serum and BAM. Outbred CD-1 mice were purchased from Charles River Laboratories, Wilmington, Mass., and inbred BALB/cAn/N/Sim (referred to hereafter as BALB/c) mice were supplied by Simonsen Laboratories, Gilroy, Calif.

BAM. Mice anesthetized with ethyl ether were exsanguinated by severing of the brachial artery. Blood was collected as a source of fresh mouse serum. After cervical dislocation, pulmonary lavage was performed as described by Sugar et al. (21). Briefly, 10 ml of phosphate-buffered saline with 0.1% EDTA (free acid) adjusted to pH 7.4 was used to lavage the lungs of each mouse. Cells were pelleted by centrifugation (225 × g, 10 min). Pelleted cells from five or more mice were pooled, washed, and counted. Cells were suspended in complete tissue culture medium (CTCM) consisting of RPMI 1640, 10% (vol/vol) heat-inactivated fetal bovine serum, and penicillin (100 U/ml) plus streptomycin (100 μg/ml). Cells at 1.5 × 10⁶/ml of CTCM were dispensed at 0.1 ml per well of an A2 microtiter plate (Costar, Cambridge, Mass.). After 2 h at 37°C with 5% CO₂ plus 95% air, nonadherent cells were aspirated and adherent cell monolayers (BAM) were washed once with RPMI 1640. Approximately 90% of plated cells were adherent and had alveolar macrophage morphology.

C. neoformans. Three encapsulated isolates of C. neoformans were used in these studies. The capsule thickness ranged from 1.0 to 1.3 μm. Susceptibility testing performed by broth dilution methods previously described (13) indicated that isolate CDC 9759 and a clinical isolate, W, were susceptible to FCZ. Isolate 92-197 was classified as resistant. Susceptibility...
was defined, on the basis of achievable concentrations in serum, as the possession of a MIC of 12.5 μg/ml or less, and resistance was defined as possession of a MIC of 100 μg/ml. C. neofor manis was grown for 48 h at 37°C on blood agar plates. Yeast cells were washed with saline, counted, and suspended in CTCM plus 10% (vol/vol) fresh mouse serum as the source of complement for opsonization.

**Treatment of BAM.** BAM monolayers were treated with CTCM or 5,000 U of human recombinant M-CSF (Genetics Inst., Cambridge, Mass.; 1.9 × 10⁹ U/mg) per ml for increasing periods of time.

**Fungicidal and fungistatic assays.** BAM cultured in CTCM or M-CSF were challenged with 0.1 ml of C. neofor manis in CTCM plus 10% fresh mouse serum for 3 h in a fungicidal assay. At time zero, the inoculum was plated to determine the number of CFU per well. After the 3 h at 37°C in 5% CO₂ plus 95% air, the control culture (yeast cells alone) and the experimental macrophage cocultures were harvested with diluted water to lyse macrophages. Dilutions of harvested material were plated on blood agar plates, and CFU were counted after 48 h at 35°C. Microscopic examination of microtiter wells revealed the removal of macrophages from the wells. Microscopic examination of the harvested material showed that the macrophages were lysed and there was no clumping of yeast cells. The percent fungicidal activity was determined as [1 – (experimental CFU/inoculum CFU)] × 100. The same procedure was used in 24-h fungistatic assays, and percent fungistasis was calculated as [1 – (experimental CFU/CFU in 24-h CTCM)] × 100.

**Fungicidal mechanisms.** Superoxide and hydrogen peroxide scavengers, superoxide dismutase and catalase (Sigma Chemical Co., St. Louis, Mo.), were used to see if killing was mediated by products of the oxidative burst. Reagents were prepared, and concentrations were used as previously reported (5, 7). N⁵-monodemethyl-L-arginine (NMMA) was used as the competitive analog inhibitor of L-arginine, the substrate for the production of nitric oxide by activated macrophages (6, 8).

**Statistics.** Comparison between groups was made by Student’s t test with the significance set at P < 0.05.

**RESULTS**

**Effect of culture time on BAM fungicidal activity.** BAM from BALB/c mice cultured for 24 h in CTCM or M-CSF (5,000 U/ml) were not fungicidal against C. neofor manis in a 3-h assay. However, after 48 h of culture in CTCM or M-CSF (5,000 U/ml), BAM killed yeast cells at 24 h (P < 0.01) and 45% (P < 0.001), respectively (Fig. 1). M-CSF BAM were significantly (P < 0.05) more fungicidal than CTCM BAM in this experiment (Fig. 1). When BAM were cultured for 72 h in CTCM or M-CSF (5,000 U/ml), they did not have significantly increased fungicidal activity.

**Effect of culture time on fungistatic activity of BAM.** BALB/c BAM cultured for 24 h in CTCM or M-CSF (5,000 U/ml) and then challenged for 24 h significantly (P < 0.001) and equally inhibited the growth of C. neofor manis (Fig. 2). Fungistatic activity was significantly increased (P < 0.001) if BAM were cultured for 48 h in CTCM or M-CSF before challenge, to 98 and 99%, respectively (Fig. 2). If culture time was extended to 72 h before challenge, fungistasis of CTCM BAM did not significantly change; however, M-CSF BAM were then fungicidal in the 24-h assay (Fig. 2).

**Effect of challenge inoculum size on BAM antifungal activity.** BALB/c BAM were cultured for 48 h in CTCM or M-CSF and then challenged with increasing doses of C. neofor manis for 3 h. Both CTCM and M-CSF BAM were significantly fungicidal, at 21 (P < 0.01) and 30% (P < 0.001), respectively, against the low inoculum (Fig. 3). When the challenge dose was increased threefold, there was no significant killing by CTCM or M-CSF BAM; however, both had significant fungistasis (P < 0.01) (Fig. 3). At the highest challenge dose, eight times the lowest dose, CTCM BAM were still fungistatic (P < 0.05) and M-CSF BAM were modestly fungicidal (17%, P < 0.01) (data not shown).

**Fungidical mechanisms.** The presence of superoxide dismutase (500 U/ml) or catalase (20,000 U/ml) during the 3-h fungidical assay did not inhibit killing (29%) by M-CSF BALB/c BAM. The presence of NMMA (0.2 mM) during the killing assay also had no effect on killing. These results suggest that killing was not mediated by products of the oxidative burst or generation of nitric oxide in this time frame.

**Effects of mouse strain and C. neofor manis isolate on killing**

FIG. 1. Effects of treatment time on BAM killing of C. neofor manis. Quadruplicate cultures of BAM were incubated with medium or M-CSF (5,000 U/ml) for 1, 2, or 3 days and then challenged with C. neofor manis CDC 7959 for 3 h. CFU counts (means ± standard deviations [SD]) (n = 4) of the inoculum at 0 h and the inoculum after 3 h (top two lines) are shown; below these, counts of cocultures after 3 h (results in pairs: those for the controls [BAM in medium] above and the corresponding M-CSF values below) are shown. Killing (reduction of inoculum CFU in 3 h by BAM previously cultured for 1, 2 or 3 days [numbers in parentheses]) is depicted.

![FIG. 1. Effects of treatment time on BAM killing of C. neofor manis.](http://aac.asm.org/)

FIG. 2. Effect of culture time of BAM on fungistasis against C. neofor manis. Quadruplicate cultures of BAM were incubated with medium or M-CSF (5,000 U/ml) for 1, 2, or 3 days (numbers in parentheses) and then challenged with C. neofor manis CDC 7959 for 24 h. CFU counts (means ± standard deviations [SD]) (n = 4) of the inoculum at 0 h, of the inoculum after 24 h, and of cocultures after 24 h are given. Fungistasis (inhibition of growth in cocultures compared with growth in medium alone) is shown.

![FIG. 2. Effect of culture time of BAM on fungistasis against C. neofor manis.](http://aac.asm.org/)
by BAM. BAM from CD-1 and BALB/c mice cultured for 72 h in CTCM or M-CSF (5,000 U/ml) had similar fungicidal activities against isolate CDC 9759 (Table 1). Likewise, when isolate W was tested against CD-1 and BALB/c BAM cultured in CTCM or M-CSF, killing of this isolate was slightly higher but similar for all BAM (Table 1).

Synergy of BAM with FCZ for killing. FCZ at 2.5 and 5.0 μg/ml was fungistatic against C. neoformans CDC 9759 in 24- and 48-h assays (Table 2). CTCM and M-CSF BALB/c BAM were also fungistatic in 24-h (87% and 98%, respectively) and 48-h (66% and 98%, respectively) assays.

Fungistatic BALB/c BAM and FCZ synergized for killing C. neoformans in 24-h cultures. M-CSF BAM were more efficient than CTCM BAM in acting synergistically with FCZ at 2.5 μg/ml for killing, e.g., 55% versus 20%, P < 0.05 (Table 2). There was no significant difference between M-CSF BAM and CTCM BAM in their synergy with FCZ at 5.0 μg/ml for killing at 24 h.

In 48-h cultures, M-CSF BAM were more fungistatic than CTCM BAM (98% versus 66%, P < 0.001). Moreover, M-CSF BAM had a greater synergy with FCZ at 2.5 μg/ml than did CTCM BAM for killing (96% versus 29%, P < 0.01) (Table 2).

**TABLE 1.** Effects of mouse strain and C. neoformans isolate on killing by BAM

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Treatment of BAM</th>
<th>% Killing (mean ± SD) of indicated isolate</th>
<th>p&lt;sup&gt;±&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CDC 9759</td>
<td>W</td>
<td></td>
</tr>
<tr>
<td>CD-1</td>
<td>Medium</td>
<td>28 ± 3</td>
<td>35 ± 1</td>
</tr>
<tr>
<td></td>
<td>M-CSF</td>
<td>24 ± 3</td>
<td>41 ± 7</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Medium</td>
<td>25 ± 4</td>
<td>47 ± 7</td>
</tr>
<tr>
<td></td>
<td>M-CSF</td>
<td>25 ± 2</td>
<td>41 ± 9</td>
</tr>
</tbody>
</table>

<sup>±</sup> Quadruplicate cultures of BAM were incubated for 3 days in medium alone or medium plus M-CSF (5,000 U/ml) and then challenged with C. neoformans for 3 h.

<sup>±</sup> Means for three experiments.

<sup>±</sup> P value in comparison of differences in percent killing of CDC 9759 and W by CD-1 or BALB/c BAM.

<sup>±</sup> NS, not significant (P > 0.05).

The same was true for M-CSF BAM versus CTCM BAM with FCZ at 5.0 μg/ml (96% versus 80% killing, P < 0.01) (Table 2). These findings indicate that M-CSF significantly enhances BAM fungistasis and synergy with FCZ for killing.

Results similar to those above were obtained in a second experiment. For example, M-CSF BAM were significantly more fungistatic against C. neoformans CDC 9759 than were CTCM-cultured BAM at 24 and 48 h (P < 0.001). Killing by BAM plus FCZ (5.0 μg/ml) significantly increased from 50% in 24 h to 92% in 48 h (P < 0.001). Killing by M-CSF BAM plus FCZ (5.0 μg/ml) also significantly increased from 76% to 24 h to 94% at 48 h (P < 0.001).

**TABLE 2.** Synergy of BAM and FCZ in killing of C. neoformans in 24- and 48-h assays

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>No. of CFU (mean ± SD) with indicated concn (μg/ml) of FCZ at indicated time&lt;sup&gt;±&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Medium alone 114,000 ± 3,000 6,800 ± 970 [94] 2,035 ± 415 [98]</td>
</tr>
<tr>
<td></td>
<td>BAM 15,400 ± 600 [87] 480 ± 125 [20] 360 ± 160 [40]</td>
</tr>
<tr>
<td></td>
<td>M-CSF 2,325 ± 395 [98] 270 ± 76 [55] 280 ± 20 [54]</td>
</tr>
<tr>
<td>48</td>
<td>Medium alone 313,000 ± 66,000 59,000 ± 6,200 [81] 2,900 ± 1,150 [99]</td>
</tr>
<tr>
<td></td>
<td>BAM 107,500 ± 14,000 [66] 430 ± 205 [29] 120 ± 40 [80]</td>
</tr>
</tbody>
</table>

<sup>±</sup> BAM were cultured for 2 days with medium or medium plus M-CSF (5,000 U/ml) prior to challenge.

<sup>±</sup> The initial inoculum (0 h) was 600 ± 52 CFU. Means are for quadruplicate cultures. The percent fungistasis is indicated in parenthesis; the percent killing is indicated in brackets.

**DISCUSSION**

The modest killing of C. neoformans by BAM from outbred CD-1 mice reported here is similar to that found by Levitz and DiBenedetto (14). The fungicidal activity of BAM against C. neoformans noted here is similar to the killing of Candida parapsilosis by BAM (4). By contrast, killing activity by BAM against Candida albicans is only slight in this system.

BAM from inbred BALB/c mice were fungicidal against C. neoformans after being cultured in tissue culture medium for 2 to 3 days. We speculate that adherence, factors in culture medium, and M-CSF endogenously produced by BAM could be factors in BAM acquisition of fungicidal activity. On the other hand, exogenous M-CSF was found to enhance fungicidal activity compared with BAM cultured in tissue culture
medium. In previous work, we found that peritoneal macrophages, but not BAM, could be activated by gamma interferon in 24 h for killing of *C. albicans*. However, BAM could be activated to kill *C. albicans* by supernatants from concanavalin A-stimulated spleen cells (4). The BAM-activating factor(s) in concanavalin A supernatants have not been identified.

Significant fungicidal activity of BAM was found to be dependent on a high BAM-to-yeast cell ratio. This is a situation likely to be found in a natural pulmonary exposure. Furthermore, we found as did others that the fungicidal activity of BAM against *C. neoformans* did not depend on products of the oxidative burst or on nitric oxide production (14).

Even though BAM were only modestly fungicidal against *C. neoformans*, they were strongly fungastatic against surviving yeast cells in 24-h assays. In this respect, BAM activities were similar to the anticyptococcal activity of human alveolar macrophages (24). M-CSF could significantly enhance the fungistatic activity of BAM compared with control BAM. It remains to be seen if M-CSF could have a similar effect on human alveolar macrophages.

BAM from CD-1 and BALB/c mice cultured for 3 days in tissue culture medium or M-CSF had similar fungastatic activities against isolates of *C. neoformans*. Both M-CSF and control BAM killed the more slowly growing isolate W more efficiently than the faster-growing CDC 9795 isolate. Although lower growth rate correlated with susceptibility, it is possible that other characteristics of isolate W were responsible for greater susceptibility. Others have found an association between greater encapsulation and enhanced killing of opsonized *C. neoformans* by BAM (14). Both isolates tested here had medium-sized capsules; therefore, differences in susceptibility were not due to capsule size.

FCZ at pharmacological concentrations is only fungastatic against *C. neoformans* in vitro. However, we show here that these concentrations of FCZ synergize with fungastatic BAM for efficient killing of *C. neoformans*. FCZ can also act synergistically with peritoneal macrophages for killing FCZ-sensitive isolates of *C. neoformans* (6). These findings suggest an explanation for the in vivo efficacy of FCZ in cryptococcosis. The synergistic mechanism by which *C. neoformans* is killed is not known at this time.

On the other hand, BAM and peritoneal macrophages (6) cannot synergize with an FCZ-resistant isolate for killing. These findings suggest a possible new relevance for susceptibility testing of *C. neoformans* isolates.

Since M-CSF enhances macrophage fungastasis and synergy with FCZ in killing *C. neoformans*, therapeutic applications of M-CSF as an adjunct to antifungal therapy merit consideration.

REFERENCES


