Killing of *Histoplasma capsulatum* by macrophage colony stimulating factor-treated human monocyte-derived macrophages: role for reactive oxygen intermediates

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**Summary.** The interaction of human macrophages with the yeast-form of *Histoplasma capsulatum* was studied. The use of culture and a short-term assay period instead of microscopy gave direct evidence of the fungicidal activity of human macrophages. The present study reports the novel finding of fungicidal activity of macrophages derived from monocytes in the presence of macrophase colony-stimulating-factor (MCSF). The induction of fungicidal activity by this cytokine was dose dependent. MCSF at 10000 U/ml was optimal with 73(SD3)% killing. Inhibition of macrophage killing by superoxide dismutase (SOD), but not catalase (CAT) or N-monomethyl-L-arginine (NMMA), established the role of the superoxide anion in the killing mechanism. The fungistatic activity of MCSF-derived human macrophages in a 24-h assay was also dose dependent and was not inhibited by SOD, CAT or NMMA. MCSF at 10000 U/ml produced optimal macrophage fungistatic activity, 34±6(SD4)%.

### Introduction

*Histoplasma capsulatum*, a thermally dimorphic fungal pathogen, causes histoplasmosis in man. Infection is established by inhalation of conidia from the soil-inhabiting saprophytic mycelial stage. In the lungs, conidia transform into the parasitic yeast form, which causes disease that ranges from a benign infection to chronic cavitation or disseminated infection. Reactivation of previously controlled histoplasmosis has recently become a serious problem in patients with the acquired immune deficiency syndrome (AIDS).1,2

*H. capsulatum* yeasts survive and multiply in human macrophages.3 However, there is evidence that cytokine-treated macrophages inhibit intracellular multiplication.4 To further define the role for human macrophages in histoplasmosis, the interaction of macrophages derived from monocytes, in the presence or absence of macrophage colony-stimulating-factor (MCSF), and *H. capsulatum in vitro* was studied. A coculture technique previously developed for the study of human macrophages and *H. capsulatum* was used.5

### Materials and methods

#### Monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised (30 U/ml) blood from healthy human donors, without any history of histoplasmosis, by density gradient centrifugation (Histopaque, Sigma). The PBMC were washed twice in RPMI-1640 (Bio-Whittaker Lab., Walkerville, MD, USA). The PBMC were then adjusted to 5×10⁶ cells/ml in complete tissue culture medium (CTCM) (RPMI-1640 with penicillin 100 U/ml, streptomycin 100 μg/ml and fresh autologous serum 10%). Briefly, 0.25 ml of the suspension of PBMC in CTCM was dispensed into each chamber of eight-chamber Lab-Tek chamber slides (Nunc Inc., Naperville, IL, USA), and incubated for 2 h at 37°C in CO₂ 5% and air 95%. After incubation, the non-adherent cells were removed by aspiration and each well was washed once with RPMI-1640. The number of adherent cells per well comprised c. 19% of the PBMC (9.5×10⁴ cells/chamber).

#### Treatment of monocytes

Duplicate wells of adherent PBMC were cultured in CTCM or CTCM containing increasing concentra-
tions of human recombinant M-CSF (specific activity
1.9 x 10^6 U/mg of protein; Genetics Institute,
Cambridge, MA, USA). After incubation for 5 days at
37°C in air 95 % and CO2 5 %, culture supernates
were aspirated and monolayers of monocyte-derived
macrophages were tested for fungicidal or fungistatic
activity against H. capsulatum. Preliminary
experiments showed that a 5-day treatment with
M-CSF was optimal for induction of killing activity.

H. capsulatum

A well characterised isolate of H. capsulatum
(G217B, ATCC 26032) was used. The organism was
grown on supplemented brain heart infusion (S-BHI)
agar plates at 37°C. S-BHI consisted of 445 ml of BHI
agar with 50 ml of bovine serum albumin 1 % and
10 ml of a H. capsulatum culture filtrate as reported by
Kwon-Chung and Tewari.6 Fresh yeast cells, harvested
after growth for 2 days, were washed with saline, and
fungal units were counted in a haemocytometer. The
morphology of fungal units was characterised as to the
number of yeast cells per unit, i.e., 49 % of the fungal
units consisted of one yeast cell, 33 % had two yeast
cells, 12 % contained three yeast cells and 6 % con-
sisted of four yeast cells. H. capsulatum was then
suspended in CTCM.

Fungicidal assay

After the 5-day treatment period, monolayers of
macrophages were challenged with 0.25 ml of yeast cell
suspension. At the time of challenge, scavengers of
superoxide anion (superoxide dismutase; SOD), H2O2
(catalase; CAT) or a competitive inhibitor of L-
arginine (NMMA) were added to different sets of
wells. After incubation for 3 h at 37°C in CO2 5 %,
non-adherent H. capsulatum cells were aspirated and
the monolayer was washed once with RPMI-1640. The
aspirate plus the rinse material was cultured to
determine the number of non-adherent fungal units.
Macrophage monolayers with ingested or adherent
fungal units were harvested with five washes of sterile
water to lyse macrophages. Different dilutions of
harvested materials were plated on S-BHI agar plates.
The plates were dried for 1 day at 35°C and then
incubated at 37°C for 5-7 days at 5 %, 10 %, and
24-h cultures. Duplicate recordings of macrophages with ingested yeast cells from duplicate
chambers, 100 macrophages per recording (n = 4)
were used to calculate the mean and SD of yeast
cells/macrophage.

Fungistatic assay

After the challenge and exposure to inhibitors of
fungicidal activity, co-cultures were incubated for
24 h. Non-adherent and adherent-ingested yeast cells
in monolayers were collected as before. The harvested
material was plated on S-BHI plates and incubated at
37°C for 5-7 days. The number of cfu/culture was
calculated and fungistasis was determined by the
formula: [1 - (cfu from experimental culture/cfu from
control culture)] × 100.

Statistical analysis

Comparison between groups was made by Student’s
t test.

Results

Effect of M-CSF on fungicidal activity of macrophages

When monocytes were cultured for 5 days in the
presence of increasing concentrations of M-CSF (1000,
5000, 10000 U/ml), fungicidal activity for H.
capsulatum increased in a dose-dependent manner
(table I). Macrophages derived from cells treated
with M-CSF 5000 U/ml had significant killing
(46 SD 15 %) (p < 0.02) compared to control macro-
phages. The killing by macrophages derived from
cells treated with M-CSF 10000 U/ml was significantly
increased (73 SD 3 %) (p < 0.05) compared to killing
by macrophages derived from cells treated with M-CSF
5000 U/ml (table I).

Killing of adherent-ingested H. capsulatum by M-CSF
macrophages

When the number of non-adherent cfu of H.
capsulatum was determined there was no significant
difference between the experimental groups (fig. 1).
In contrast, the number of cfu from the adherent-ingested
yeast cells in M-CSF-derived macrophages decreased
in an M-CSF dose-dependent manner (fig. 1, table I).
This indicates that the major effect of M-CSF on
macrophages was killing of adherent-ingested yeast
cells. The killing by macrophages treated with M-CSF
5000 U/ml was 55 % (p < 0.02). Reduction of
adherent-ingested cfu from MCSF-activated macrophage co-cultures was not due to clumping of yeast cells in harvested material that was plated. Microscopic examination of harvested material showed that the morphology of fungal units was similar to that of the inoculum.

**Killing mechanism of MCSF-activated macrophages**

When MCSF-derived macrophages were challenged with *H. capsulatum* in the presence of SOD, CAT or NMMA, it was found that only SOD significantly inhibited killing by 38% (p < 0.05) (table II). In two other experiments, SOD reduced killing by MCSF-activated macrophages from 75(SD4)% to 53(SD2)% (p < 0.05) fungistatic activity with increasing concentration of MCSF (table III). On increasing the dose of MCSF from 5000 to 10000 U/ml, fungistasis was increased from 25(SD1)% to 34(SD4)% (p < 0.05).

**Fungistasis of adherent-ingested *H. capsulatum* by MCSF-activated macrophages**

When the number of non-adherent cfu of *H. capsulatum* was determined there was no significant difference between experimental groups (fig. 2). In contrast, the number of cfu from adherent-ingested yeast cells was decreased by MCSF-activated macrophages when compared to control macrophages.

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**Table I. Effect of MCSF on fungicidal activity of macrophages for *H. capsulatum***

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Mean (SD) killing (%)</th>
<th>Number of experiments</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCM (control)</td>
<td>12 (18)</td>
<td>6</td>
<td>...</td>
</tr>
<tr>
<td>MCSF 1000 U/ml</td>
<td>42 (19)</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>MCSF 5000 U/ml</td>
<td>46 (15)</td>
<td>3</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>MCSF 1000 U/ml</td>
<td>73 (3)</td>
<td>4</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* Macrophages were derived from monocytes by incubation with CTCM or MCSF for 5 days.

**Table II. Killing mechanism of MCSF-activated macrophages for *H. capsulatum***

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Scavengers or antagonists†</th>
<th>Mean (SD) cfu (n = 4)</th>
<th>Percentage killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCM (control)</td>
<td>...</td>
<td>5360 (1032)</td>
<td>3</td>
</tr>
<tr>
<td>MCSF</td>
<td>...</td>
<td>1620 (506)</td>
<td>71</td>
</tr>
<tr>
<td>MCSF</td>
<td>SOD</td>
<td>3748 (1304)</td>
<td>33†</td>
</tr>
<tr>
<td>MCSF</td>
<td>CAT</td>
<td>1584 (600)</td>
<td>71</td>
</tr>
<tr>
<td>MCSF</td>
<td>NMMA</td>
<td>1310 (233)</td>
<td>76</td>
</tr>
</tbody>
</table>

† Superoxide dismutase (SOD) 500 U/ml, catalase (CAT) 20000 U/ml or N-monomethyl-L-arginine (NMMA) 0.2 mM added at the time of challenge.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Mean (SD) fungistasis (%)</th>
<th>Number of experiments</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCM (control)</td>
<td>1.7 (3.5)</td>
<td>4</td>
<td>...</td>
</tr>
<tr>
<td>MCSF (5000 U/ml)</td>
<td>25(10)</td>
<td>2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MCSF (10000 U/ml)</td>
<td>34(4)</td>
<td>3</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* Macrophages were derived from monocytes by incubation with CTCM or MCSF for 5 days.

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**Fungistasis of *H. capsulatum* by MCSF-activated macrophages**

MSCF-derived macrophages challenged with *H. capsulatum* for 24 h showed significantly increased (p < 0.001) fungistatic activity with increasing concentration of MCSF (table III). On increasing the dose of MCSF from 5000 to 10000 U/ml, fungistasis was increased from 25(SD1)% to 34(6)(SD4)% (p < 0.05).
KILLING OF *H. CAPSULATUM* BY MCSF-TREATED MACROPHAGES 227

![Graph](image)

**Fig. 2.** Fungistasis of adherent-ingested *H. capsulatum* by MCSF-derived macrophages. Non-adherent-non-ingested (NAD) and adherent-ingested (AD) mean cfu, of *H. capsulatum* from 24-h co-cultures of macrophages (MP) derived in 5 days in medium (CTCM MP) or MCSF 5000 U/ml (MCSF MP) are shown. NAD and AD mean cfu, of *H. capsulatum* from 24-h co-cultures of MCSF MP to which catalase (CAT) 20000 U/ml (MCSF MP + CAT) or N-monomethyl-L-arginine (NMMA) 0.2 mM (MCSF MP + NMMA) had been added. Mean of four experiments and SD shown.

**Table IV.** Inhibition of intracellular multiplication of ingested *H. capsulatum* by MCSF-activated macrophages

<table>
<thead>
<tr>
<th>Treatment of monocytes for 5 days</th>
<th>Mean number of yeast cells (SD)/macrophage after 3 h</th>
<th>Mean number of yeast cells (SD)/macrophage after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCM (control)</td>
<td>1.9 (0.1)</td>
<td>5.5 (0.5)</td>
</tr>
<tr>
<td>MCSF 5000 U/ml</td>
<td>23 (0.5)*</td>
<td>42 (0.1)†</td>
</tr>
</tbody>
</table>

* Not significant from control (*p* > 0.05) at 3 h.
† *p* < 0.01, compared to control at 24 h.

Inhibition of intracellular multiplication of ingested *H. capsulatum* by MCSF-activated macrophages

Macrophages treated with MCSF showed a significant decrease in the average number of yeast cells/macrophage at 24 h compared to the control group (42 SD0:1 versus 5.5 SD0:05; *p* < 0.01; table IV). No significant difference was seen at baseline in the mean number of yeast cells/macrophage between control and MCSF-treated groups.

Microscopic studies

Compared to the morphology of CTCM monocyte-derived macrophages (not shown), MCSF-treated monocyte-derived macrophages (5000 U/ml, fig. 3A; 10000 U/ml, fig. 3B) had a differentiated morphology as demonstrated by increased size and spreading. Macrophages from monocytes treated with MCSF at 10000 U/ml (fig. 3B) were distinctly larger and more elongated than those from monocytes treated with 5000 U/ml (fig. 3A). The number of yeast cells/macrophage after a 2-h ingestion period (fig. 3A) illustrates the starting point in a challenge experiment. Groups of yeast cells in macrophages after incubation on challenging MCSF (5000 U/ml)-activated macrophages in the presence of a potential antagonist (NMMA), fungistasis was not abrogated (fig. 2). However, CAT had some inhibitory effect on fungistasis, but this was not significant. In two other experiments, where macrophages were activated with MCSF, 10000 U/ml, CAT and SOD failed to inhibit fungistatic activity significantly. MCSF-activated macrophage fungistasis was 33(SD5)% in the presence of CAT it was 38(SD5)% and in the presence of SOD it was 57(SD13)%.

On challenging MCSF (5000 U/ml)-activated macrophages in the presence of a potential antagonist (NMMA), fungistasis was not abrogated (fig. 2). However, CAT had some inhibitory effect on fungistasis, but this was not significant. In two other experiments, where macrophages were activated with MCSF, 10000 U/ml, CAT and SOD failed to inhibit fungistatic activity significantly. MCSF-activated macrophage fungistasis was 33(SD5)% in the presence of CAT it was 38(SD5)% and in the presence of SOD it was 57(SD13)%.
Discussion

The present study demonstrates the ability of MCSF to activate human macrophages, during in-vitro differentiation from monocytes, to kill H. capsulatum yeast cells in a dose-dependent manner. The fungicidal activity of activated human macrophages for H. capsulatum was demonstrated by: firstly, the use of a short-term 3-h assay period which was less than the doubling time of H. capsulatum, and secondly by the use of a culture methodology for cfu determination, demonstrating killing of H. capsulatum during a 3-h incubation period that would go undetected by microscopic techniques. This demonstration brings a better understanding of host resistance to H. capsulatum.

The ability of SOD (scavenger of O$_2^-$) to inhibit the killing of adherent-ingested yeast cells by human macrophages provides evidence for a superoxide-dependent mechanism of MCSF stimulation. The evidence of a superoxide anion-dependent mechanism for the killing of H. capsulatum by γ-interferon-activated human macrophages has been reported previously. It is unlikely that the nitric oxide-dependent pathway, reported to be involved in the killing of several intracellular parasites by mouse peritoneal macrophages, is involved in the killing of H. capsulatum because NMMA, a competitive inhibitor of the substrate for nitric oxide synthase, did not inhibit killing of H. capsulatum by MCSF-activated human macrophages. Catalase, a scavenger of H$_2$O$_2$, also had no effect on killing. These results demonstrate a superoxide anion-dependent histoplasmacidal mechanism in macrophages.

Treatment with MCSF for 5 days during differentiation from monocytes enhanced the fungistatic activity of human macrophages in a dose-dependent manner. Similar results have been reported by Newman and Gootee. No significant role for SOD, NMMA or CAT in the inhibition of fungistatic activity of activated human macrophages could be demonstrated.

In the present study, inhibition of multiplication or fungistasis was determined by a 24-h culture method where cfu were enumerated. Other studies have used an indirect method of measuring the inhibition of yeast cell multiplication, where yeast cells were labelled with $^3$H-leucine during co-culture with macrophages. Results were similar to those reported here.

The monolayer microscopic method for studying intracellular multiplication of H. capsulatum yeast cells demonstrated that MCSF-activated macrophages significantly inhibited multiplication of ingested yeast cells. Inhibition of intracellular multi-
plication of ingested yeast cells has also been reported with macrophages cultured in medium and then incubated (as co-cultures) with lymphokines for 24 h. Consequently, macrophages can be induced to an anti-histoplasmal state by more than one set of inducing conditions.

The intracellular restriction of _H. capsulatum_ multiplication by MCSF monocyte-derived macrophages reported here is similar to that reported with murine cells. Peritoneal macrophages (PM) incubated with MCSF (5000 U/ml) for 2 days significantly inhibited the intracellular multiplication of _H. capsulatum_. Likewise, MCSF treatment of PM induced dramatic increases in macrophage size and spreading. The inhibition by MCSF-cultured macrophages was equivalent to inhibition by γ-interferon (IFN)-activated macrophages.

Recently, it has been demonstrated that bronchoalveolar macrophages (BAM) cultured with MCSF (5000 U/ml) significantly restricted intracellular multiplication of _H. capsulatum_. In contrast, NMMA inhibited the anti-histoplasmal activity of BAM 3 days after treatment.

MCSF treatment of BAM or PM also induces antifungal activity for _Cryptococcus neoformans_ and enhances macrophage synergy with an antifungal drug, fluconazole, for killing. Furthermore, in-vivo administration of MCSF (2.5 mg/kg s.c.) significantly increases the fungistatic activity of BAM for _C. neoformans_ 1 and 3 days after treatment.

In summary, macrophages derived from monocytes in the presence of MCSF kill yeast cells of _H. capsulatum_ by a superoxide-dependent mechanism. Since MCSF serum levels increase during infections, it is likely that MCSF has a natural function in increasing host resistance by enhancing the antimicrobial activity of macrophages. Immunomodulation in fungal disease is of increasing clinical importance, and is relevant to several infections.

References