MYCOLOGY

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

GOPI DESAI*, F. NASSAR†, E. BRUMMER*† and D. A. STEVENS*‡

*Division of Infectious Disease, Department of Medicine, Santa Clara Valley Medical Center and California Institute for Medical Research, 751 South Bascom Avenue, San Jose, CA 95128 and †Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University, Stanford, CA 94305, USA

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 macrophage 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-
KILLING OF H. CAPSULATUM BY MCSF-TREATED MACROPHAGES

Assessment of intracellular multiplication of H. capsulatum

Duplicate macrophage monolayer cultures established in Lab-Tek chambers were challenged with H. capsulatum as described above. After incubation for 3 h to allow ingestion, sets of monolayers were washed with phosphate-buffered saline (PBS), dried and stained with Diff-Quik (Baxter Corporation, McGaw Park, IL, USA) for baseline data. Other sets of washed cultures were incubated with CTCM for 24 h and processed for microscopic examination as above. The number of yeast cells/macrophase was recorded in control and experimental monolayers for 3-h (baseline) 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/macrophase.

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 (NMMMA) 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 phosphate-buffered saline (PBS). 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 (NMMMA) 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.

Results

Effect of MCSF on fungicidal activity of macrophages

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

Killing of adherent-ingested H. capsulatum by MCSF 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 MCSF-derived macrophages decreased in an MCSF dose-dependent manner (fig. 1, table I). This indicates that the major effect of MCSF on macrophages was killing of adherent-ingested yeast cells. The killing by macrophages treated with MCSF 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 11(SD15)%. Heated SOD (120°C, 15 min) had no significant effect on killing by MCSF-activated macrophages.

**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).

**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.
KILLING OF H. CAPSULATUM BY MCSF-TREATED MACROPHAGES 227

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>19 (0-1)</td>
<td>55 (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 (4.2 SD ± 1 versus 5.5 SD ± 0.5; 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 (SD ± 5)% in the presence of CAT it was 38 (SD ± 5)% and in the presence of SOD it was 57 (SD ± 13)%.
Fig. 3. Dose- and time-dependent morphological changes in MCSF-derived macrophages. A, Macrophages derived for 5 days in 5000 U/ml MCSF with ingested yeast cells (arrows) after a 2-h ingestion period. B, Macrophages derived for 5 days in 10000 U/ml MCSF were allowed to ingest yeast cells, then incubated in medium for 24 h. The macrophages show increased size and there is some intracellular multiplication of yeast cells (arrows).

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 O₂⁻) 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 H₂O₂, 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 ³¹H-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 \textit{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 \textit{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 \(\gamma\)-interferon (IFN)–activated macrophages.

Recently, it has been demonstrated that bronchoalveolar macrophages (BAM) cultured with MCSF (5000 U/ml) significantly restricted intracellular multiplication of \textit{H. capsulatum}, without inducing dramatic morphological changes in BAM. The anti-histoplasmal activity of MCSF-treated murine BAM or PM could not be abrogated by NMMA. In this respect murine macrophages treated with MCSF resembled human MCSF monocyte-derived macrophages. In contrast, NMMA inhibited the anti-histoplasmal activity of IFN plus lipopolysaccharide (LPS) activated murine BAM and PM. Moreover, MCSF given subcutaneously (2.5 mg/kg) significantly increased the anti-histoplasmal activity of BAM 3 days after treatment.

MCSF treatment of BAM or PM also induces antifungal activity for \textit{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 \textit{C. neoformans} 1 and 3 days after treatment.

In summary, macrophages derived from monocytes in the presence of MCSF kill yeast cells of \textit{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