Killing of *Histoplasma capsulatum* by γ -interferon-activated human monocyte-derived macrophages: evidence for a superoxide anion-dependent mechanism

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Summary. The interaction of human macrophages with the yeast form of the thermally dimorphic fungal pathogen, *Histoplasma capsulatum*, was studied. Macrophages derived from monocytes by culture *in vitro* for 3 days ingested *H. capsulatum*, but were neither fungicidal or fungistatic. In contrast, when monocytes were exposed to human recombinant gamma-interferon (γ -IFN) during their differentiation into macrophages, those macrophages were able to reduce the number of ingested or adherent cfu of *H. capsulatum* by 44–75% in 2 h. Activation of macrophages for fungicidal activity by γ -IFN was dose dependent and 500–1000 units ml were optimal. Antibody to γ -IFN abrogated the γ -IFN activation process. Killing of *H. capsulatum* by activated macrophages in 2-h assays could be inhibited by superoxide dismutase but not by sodium azide.

Introduction

Histoplasma capsulatum, a thermally dimorphic fungal pathogen, is the causative agent of histoplasmosis in man.¹ Infection is established by inhalation of conidia from the soil-inhabiting saprophytic mycelial stage. In the lungs, conidia transform into the parasitic yeast form causing disease that ranges from benign, to chronic cavitating, or to disseminated infections.² Reactivation of previously controlled histoplasmosis has recently become a serious problem in patients with the acquired immunodeficiency syndrome (AIDS).^{3,4}

To define a possible role for human macrophages in histoplasmosis, we have studied the interaction, in the presence or absence of human recombinant gamma-interferon (γ -IFN), of macrophages derived from monocytes and *H. capsulatum* *in vitro*. Co-culture techniques developed for the study of human macrophages and *Blastomyces dermatitidis* were used.⁵

Materials and methods

Monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised (10 U/ml) blood of healthy human donors without histories of histoplasmosis. Standard methods, as previously described, were

used.⁵ Monocytes in PBMC were selected by adherence to plastic or glass during incubation at 37°C for 2 h in an atmosphere of CO₂ 5% in air. Complete tissue culture medium (CTCM) consisted of RPMI-1640 (Gibco Laboratories, Long Island, NY, USA) containing penicillin 100 μ /ml, streptomycin 100 μ /ml, and fresh autologous serum 15%. Briefly, 0.2 ml of a suspension of PBMC 3×10^6 /ml in CTCM, was dispensed into each well of flat-bottomed 96-well microtitration plates. After incubation, non-adherent cells were removed by aspiration and each well was washed once. The number of adherent cells was calculated by subtracting the mean number of non-adherent cells in each well from the original number of cells per well. Approximately 19% of PBMC were adherent to plastic, i.e., 1.2×10^5 per well, and, as was shown previously, 90% were esterase positive.⁵ For experiments with Lab-Tek chamber slides (Nunc, Inc., Naperville, IL, USA), 0.25 ml of a suspension containing PBMC 3×10^6 ml was dispensed into each chamber. Adherent cells were selected and characterised as above.

Treatment of monocytes

Quadruplicate wells of adherent PBMC were cultured in CTCM or CTCM containing increasing concentrations of human recombinant γ -IFN (Collaborative Research Inc., Bedford, MA, USA). After incubation for 3 days at 37°C in air + CO₂ 5%, culture supernates were aspirated and monolayers of monocyte-derived macrophages were tested for fungicidal or fungistatic activity against *H. capsulatum*.

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H. capsulatum

A well characterised isolate of *H. capsulatum*, CDC 105 (serotype 1,4 and chemotype 2), was used. The major wall component of this isolate consisted of α -1,3 glucan.⁶ *H. capsulatum* was grown on brain heart infusion (BHI) agar slants at 37°C. Fresh growth (3–4 days) was collected and washed with phosphate-buffered saline (PBS), and fungal units were counted in a haemocytometer. Quantitation of the inoculum according to morphology of fungal units showed that 41% SD 1% were one-cell units, 34% SD 5% were units with two cells, 11% SD 4% had three cells per unit, 7% SD 4% had four cells per unit, and 7% SD 2% consisted of units with five or more cells. The medium for cfu determinations by plating was that described by Schaffner *et al.*,⁷ except that sheep blood 10% was substituted for rabbit blood in the BHI agar plates. Preliminary experiments showed that 85% of the fungal units, as determined by haemocytometer counting, were recovered as cfu with this medium.

Fungicidal assay

After the 3-day treatment period, macrophage monolayers were challenged with *H. capsulatum*, 0.2 ml of a suspension containing 10 000 fungal units per ml of CTCM. After 2 h at 37°C in air + 5% CO₂, non-adherent *H. capsulatum* cells were aspirated and the monolayers were rinsed with 0.2 ml of CTCM. Aspirated + rinse material were cultured to determine the number of non-adherent cfu. Monolayers with ingested *H. capsulatum* were harvested with distilled water to lyse macrophages as previously described.⁵ From harvested material, 1 ml per culture was plated to each BHI blood-agar plate. Plates were incubated for 2 days at 37°C, placed in plastic containers to prevent dehydration, and incubated for 5 more days at 37°C. The numbers of cfu/plate were recorded and cfu/culture were calculated. Since there were no significant differences between the numbers of non-adherent cfu of *H. capsulatum* cultured from monolayers treated with CTCM or γ -IFN, the number of adherent or ingested cfu in control and experimental monolayers were equivalent. Based on this, the percentage killing was determined by the formula: $[1 - (\text{cfu from experimental cultures} / \text{cfu from control cultures})] \times 100$.

Assessment of ingestion and adherence

Duplicate macrophage monolayer cultures that had been established in Lab-Tek chambers were challenged with *H. capsulatum* (0.25 ml of a suspension containing 10 000 fungal units/ml of CTCM) in a calculated ratio of macrophage to *H. capsulatum* of 1:10. After 2 h at 37°C in CO₂ 5% in air, monolayers were washed with PBS, dried, and stained with Wright's stain. Macrophages were examined micro-

scopically and the percentage of ingestion + adherence was determined by the formula: (number of macrophages with ingested *H. capsulatum* / number of macrophages examined) $\times 100$; 100 macrophages in each duplicate monolayer were examined (total = 200).

Anti- γ -IFN antibody

Rabbit antibody to affinity column purified natural human γ -IFN (Genzyme Corporation, Boston, MA, USA) was used to neutralise human recombinant γ -IFN. Anti- γ -IFN antibody was added to quadruplicate cultures of γ -IFN-treated monocyte monolayers or control cultures at the beginning of the treatment period.

Scavengers and inhibitors

Superoxide dismutase (SOD) (11 500 U/mg of protein; Seikagaku Kogyo Co., Ltd, Tokyo, Japan) was dialysed against PBS, sterilised by membrane filtration (0.45- μ m pore size) and stored at 4°C. A portion of the SOD preparation was inactivated by autoclaving (121°C, 30 min). Sodium azide (0.01 M in PBS) was sterilised by filtration as above and stored at 4°C. SOD, heat-inactivated SOD, or sodium azide were added at the same time as the *H. capsulatum* challenge to macrophage cultures.

Lab-Tek cultures

As in phagocytosis studies, duplicate monocyte-derived macrophage cultures were established for determination of killing as measured by reduction of numbers of cfu of ingested or adherent *H. capsulatum*. For these experiments, and those in which an attempt was made to follow intracellular replication by microscopic examination, the macrophage to *H. capsulatum* ratio was 33:1.

Direct counts

To control for possible variation, e.g., clumping, in the composition of fungal units in control *vs* experimental cultures, direct examination of plated material was made with a haemocytometer as previously described.⁵ When pooled, pelleted, harvested material in 0.025 ml was counted with a haemocytometer, it was possible to estimate the number of fungal units and, at the same time, record the morphology of fungal units.

Statistical analysis

Comparison between groups was made by Student's *t*-test with the level of significance considered to be $p < 0.05$.

Results

Dose requirement for γ -IFN activation of macrophages

When monocytes were cultured for 3 days in higher concentrations of γ -IFN, i.e. 500 and 1000 U/ml, they became activated and they significantly reduced the number of cfu of ingested or adherent *H. capsulatum* (table I). Treatment with γ -IFN 250 U/ml did not activate macrophages to significantly reduce the inoculum number of cfu of ingested *H. capsulatum* (table I). All the macrophage monolayers were challenged with the same inoculum for 2 h. When the non-ingested or non-adherent numbers of cfu of *H. capsulatum* were determined by plating, they were not significantly different, e.g., with CTCM alone (control) 384 SD 26 with γ -IFN 250 U/ml 423 SD 19, 500 U/ml 361 SD 43, 1000 U/ml 397 SD 41. Therefore, the number of *H. capsulatum* cfu that were ingested or adherent in all of the co-cultures were equivalent. Since 54–58% fewer cfu were recovered from cultures containing activated macrophages, this reduction of recoverable cfu was interpreted as killing (table I). This interpretation was supported by elimination of other possible explanations. For example, microscopic examination of harvested culture wells showed that the harvesting procedure removed all well contents equally, and direct microscopy of harvested material showed that the average number of cells per fungal unit was equivalent in the material from different cultures (2–3 cells/unit). Therefore, neither adherence of *H. capsulatum* cells to experimental culture wells nor clumping of the yeast cells was an explanation for the reduction in numbers of cfu.

Similar results were obtained when macrophages were tested in Lab-Tek chambers instead of microtest plate wells. Macrophages derived in the presence of γ -IFN 1000 U/ml reduced the number of ingested or adherent cfu of *H. capsulatum* by 51% compared to control macrophages (4000 SD 900 vs 8100 SD 1900). Non-adherent numbers of cfu of control and experimental cultures were not significantly different.

Inoculum size and killing of *H. capsulatum* by activated macrophages

In different experiments the inoculum size varied; consequently the number of cfu ingested or adherent to macrophages varied. Within the range tested (240 SD 20–740 SD 140) the percentage killing (44–58%) of *H. capsulatum* by the constant size γ -IFN (1000 U/ml)-activated macrophage monolayers was not significantly changed (table II).

Abrogation of activated macrophage killing

The fungicidal mechanism of activated macrophages for *H. capsulatum* was investigated by the use of superoxide anion scavenger, SOD, and an inhibitor

Table I. Effect of γ -IFN concentrations on activation of macrophages

Treatment of macrophages	Mean cfu (SD) of ingested or adherent <i>H. capsulatum</i> (n = 4)	Percentage killing compared to control
CTCM (control)	240 (20)	...
γ -IFN 250 U/ml	200 (10)	17%
γ -IFN 500 U/ml	110 (20)	54%*
γ -IFN 1000 U/ml	100 (20)	58%*

* $p < 0.001$.

of the myeloperoxidase enzyme, sodium azide. SOD 500 U/ml, which did not affect ingestion of *H. capsulatum* by macrophages, inhibited the killing of *H. capsulatum* by γ -IFN activated macrophages (0% vs 50%) (table III). Heat-inactivated SOD had no effect on killing. However, 1 mM azide had no effect on γ -IFN activated macrophage killing of *H. capsulatum* (table III). These results indicate that superoxide anion, a product of the oxidative burst, was required for the fungicidal activity, whereas the myeloperoxidase- H_2O_2 -halide system was not involved.

Neutralisation of γ -IFN activation by anti- γ -IFN

Specific antibody to γ -IFN, present during the treatment period, completely blocked the activation of monocyte-derived macrophages for killing *H. capsulatum* (fig. 1). There were significantly ($p < 0.001$) fewer cfu (44%) recoverable from γ -IFN-activated macrophage cultures than from control, anti- γ -IFN, or anti- γ -IFN + γ -IFN cultures after 2 h

Table II. Effect of increasing numbers of *H. capsulatum* on macrophage killing

Mean number (SD) of ingested or adherent cfu after 2 h in cultures of macrophages treated with		Percentage killing by γ -IFN-activated macrophages
CTCM (control) (n = 4)	γ -IFN 1000 U/ml (n = 4)	
240 (20)	100 (20)	58%*
495 (80)	280 (10)	44%*
740 (140)	360 (20)	52%*

* Percentage reduction of number of cfu in γ -IFN-treated compared to CTCM-treated (control) macrophages.

Table III. Effect of SOD or azide on fungicidal activity of activated macrophages

Treatment of macrophages	Inhibitor added with challenge	Mean cfu (SD) of ingested or adherent <i>H. capsulatum</i> (n = 4)	Percentage killing in 2 h compared to control (%)
CTCM (control)	...	465 (35)	...
γ -IFN 1000 U/ml	...	235 (10)	50
γ -IFN 1000 U/ml	SOD	470 (40)	0
γ -IFN 1000 U/ml	Azide	240 (25)	49

Fig. 1. Effect of γ -IFN, antibody to γ -IFN, and time on activity of macrophages vs *H. capsulatum*. Antifungal activity of monocytes treated for 3 days with CTCM (○—○), antibody to γ -IFN (●—●), γ -IFN + antibody to γ -IFN (□—□), or γ -IFN 1000 U/ml (■—■) after 2 h of interaction with *H. capsulatum*. Growth of ingested *H. capsulatum* from 2 to 24 h is also shown for the four types of macrophage monolayers.

(fig. 1). The numbers of cfu non-adherent to control and experimental cultures were not significantly different. The time zero inoculum was the same for all cultures.

Effect of culture time

When cultures parallel to those harvested at 2 h, which would contain the same number of cfu, were incubated for another 22 h, the cfu of *H. capsulatum* that survived the initial killing by activated macrophages were able to replicate at approximately the same rate as those in control macrophage cultures (fig. 1). These findings show that, under these conditions, activated macrophages were not fungistatic.

Combined ingestion and adherence

In ingestion assays, in which the *H. capsulatum* to macrophage ratio was 10:1, each macrophage could engulf or attach many yeast cells (fig. 2, A and B). There was no significant difference in the percentage of control macrophages ingesting or attaching yeast cells (88.5%) from that of γ -IFN (250 U/ml, 94%; 500 U/ml, 93.5%; 1000 U/ml, 95.5%) -treated macrophages. Heterogeneity of γ -IFN (500 U/ml) -activated macrophages is illustrated in fig. 2. In fig. 2A a macrophage with a large spongy nucleus is shown. In fig. 2B a macrophage with a compact kidney-shaped nucleus can be seen.

Fig. 2. Combined ingestion and attachment of *H. capsulatum* by γ -IFN (500 U/ml) -activated macrophages. (A) Ingestion and attachment of *H. capsulatum* yeast cells by an activated macrophage with a spongy nucleus. (B) An activated macrophage with a compact kidney shaped nucleus with ingested and attached yeast cells.

Heterogeneity in macrophage monolayers

When an adherent population of monocytes differentiated into macrophage-like cells during culture in CTCM, a range of morphologies were seen (fig. 3A). About 50% of the cells showed enhanced spreading, but only 17% showed spindle formation and one macrophage exhibited a spongy-type nucleus (fig. 3A). Monocytes that had differentiated in the presence of γ -IFN (1000 U/ml) also showed some heterogeneity in morphology (fig. 3B). A noticeable increase in spreading and enhanced spindle formation was exhibited by 38.9% of the cells shown, whereas the other cells remained compact.

Discussion

We report here the novel finding that γ -IFN-activated macrophages were fungicidal for *H. capsulatum* *in vitro*. The significance of induction of fungicidal activity in macrophages by γ -IFN relates to basic understanding of host defence mechanisms against this facultative intracellular pathogen as well as potential immunotherapeutic strategies.

Our findings help clarify the possible role of human macrophages in controlling this infective agent. A recent study suggested that intracellular replication of *H. capsulatum* was unaffected in γ -IFN-activated macrophages,⁸ thus implying that activated macro-

phages probably do not have a protective role. However, the protocols and techniques used in those experiments were those used previously with murine macrophages⁹ (e.g., following microscopically the number of ingested yeast cells per macrophage with time, starting after the 2-h ingestion period), and were unable to detect the fungicidal activity of activated macrophages during the ingestion period, as reported here. By using a culture methodology, we were able to demonstrate killing of *H. capsulatum* during the ingestion period which would go undetected by the microscopy technique.

Observations made by us after the 2-h ingestion period by culture methodology agreed with those reported by Fleishmann *et al.*⁸ who used microscopy-surviving yeast cells replicated in activated macrophage monolayers. These results differ from those obtained with murine macrophages; e.g., murine peritoneal macrophages treated with lymphokines restricted the intracellular growth of ingested *H. capsulatum*.⁹ The assumption that all macrophages in monolayers were activated may not be justified. We noticed that macrophage monolayers were not homogeneous; some γ -IFN-treated adherent cells remained rounded whereas others exhibited spreading. Heterogeneity of macrophage monolayers may be a possible explanation for the survival and replication of some yeast cells, which might occur if they were ingested by a subpopulation of macrophages in the monolayer.

Fig. 3. Macrophage monolayers. (A) Monocytes which differentiated into macrophage-like cells during culture in CTCM. (B) Monocytes cultivated in the presence of IFN 1000 U/ml.

The importance of a role for activated macrophage killing of *H. capsulatum* in host resistance is magnified because polymorphonuclear neutrophils are not fungicidal for *H. capsulatum* *in vitro* in short-term assays⁷ (and unpublished results). Therefore, the production of γ -IFN during infection and its influence on either differentiated macrophages or monocytes differentiating under the influence of γ -IFN, could be a crucial factor in host resistance to *H. capsulatum*. These considerations are relevant to recent reports of reactivation of previously controlled histoplasmosis in patients suffering from AIDS^{3,4} and they suggest possible immunotherapeutic strategies.

Our first report here for a fungicidal mechanism against *H. capsulatum* in human phagocytes is significant because it increases our understanding of how *H. capsulatum* can be killed. Although non-activated macrophages ingesting *H. capsulatum* have been reported to generate superoxide anion as measured by nitroblue tetrazolium reduction,¹¹ such levels apparently are not sufficient for killing *H. capsulatum*. The dependence of this fungicidal mechanism on certain levels of superoxide anion coincides with the well

documented enhanced production of superoxide anion by macrophages derived from monocytes in the presence of γ -IFN 1000 U/ml,¹⁰ and suggests that the Fenton as well as the Haber-Weiss reaction may be involved. Klebanoff¹² has described how superoxide anion reacting with H_2O_2 , or when reacting ferric iron generates ferrous iron used in the Fenton reaction ($Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} OH^- + OH^\cdot$), generates hydroxyl radicals.

We have reported previously on superoxide anion-dependent fungicidal mechanisms involved in killing of *Candida parapsilosis* by normal murine peritoneal macrophages and the killing of *Candida albicans* by γ -IFN-activated murine peritoneal macrophages.¹³ In both cases, and in the present report, SOD 450 U/ml abrogated killing. Taken together, these results and the present report document the existence of a superoxide anion-dependent fungicidal mechanism in macrophages which remains to be elucidated.

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