Clin Exp Immunol 1995; **102**:65–70

Antifungal mechanisms of activated murine bronchoalveolar or peritoneal macrophages for *Histoplasma capsulatum*

E. BRUMMER & D. A. STEVENS *Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Centre, and California Institute for Medical Research, San Jose, and Stanford University School of Medicine, Stanford University, Stanford, CA, USA*

(Accepted for publication 2 June 1995)

SUMMARY

The first line of defence against natural infection by *Histoplasma capsulatum* (Hc) consists of bronchoalveolar macrophages (BAM) and an early inflammatory response in the lungs. Little is known about the interaction of BAM and Hc, consequently we studied murine BAM *in vitro* to assess their role in the pulmonary defence in histoplasmosis. A short-term 3-h assay was used to measure fungicidal activity of control BAM and interferon-gamma (IFN-γ) plus lipopolysaccharide (LPS)-activated BAM. Fungistatic activity of BAM was determined with a 24-h assay. A method devised for measuring colony-forming units (CFU) of non-ingested non-adherent and adherent ingested yeast cells of Hc in BAM cocultures was used. Activated BAM killed Hc (reduced inoculum CFU by $25 \pm 12\%$; $n = 4$). The fungicidal activity of BAM was abrogated by 0·2 mM N^G-monomethyl-L-arginine (NMMA) or catalase but not by superoxide dismutase. In fungistatic assays activated BAM inhibited multiplication of Hc by $61 \pm 4\%$ ($n = 3$) compared with cocultures with control BAM. However, Hc multiplied 100% more in control BAM cocultures than in medium alone. Data indicated that this was due to advantages that Hc has in the intracellular environment. Only NMMA inhibited fungistatic activity of activated BAM. In experiments with peritoneal macrophages (PM), results similar to those with BAM were obtained. In conclusion, activated BAM and PM kill yeast cells of Hc by a mechanism dependent on hydrogen peroxide and products of the nitric oxide synthase (NOS) pathway, whereas fungistasis depends only on products of the NOS pathway.

Keywords macrophages *Histoplasma capsulatum* fungicidal superoxide anion nitric oxide

INTRODUCTION

Histoplasmosis is caused by *Histoplasma capsulatum* (Hc), a thermally dimorphic fungal pathogen [1]. Conidia are produced by the soil-inhabiting saprophytic mycelial stage, and infection is established by inhalation of conidia. Conidia *in vivo* transform within hours into the parasitic yeast form, causing disease ranging from benign, to chronic cavitating, or to disseminated infections [2]. In patients with AIDS, primary disease as well as reactivation of previously controlled histoplasmosis has become a serious problem [3,4].

Host resistance to Hc is still not clearly defined. Murine [5] and human [6,7] polymorphonuclear leucocytes (PMNL) have been shown to be only fungistatic for Hc. Resident murine bronchoalveolar macrophages (BAM) [8] and peritoneal macrophages (PM) [9] have little antifungal activity against yeast cells of Hc. However, PM activated with interferon-gamma (IFN-γ) [10] or macrophage colony-stimulating factor (M-CSF) [11] inhibit intracellular multiplication of ingested yeast cells. On the other hand, a possible role for cytokine-activated BAM in defence against Hc has not been reported.

Since BAM are in the first line of defence against natural infection with conidia [12,13], their study should give important information about their role in host defence. In one report it was shown that BAM from bacille Calmette–Guérin (BCG)-treated mice could inhibit the growth of ingested mycelial fragments or conidia, but not the multiplication of ingested yeast cells [8].

The purpose of the present work was to see if activating signals (IFN plus lipopolysaccharide (LPS)) could activate BAM for fungicidal and/or fungistatic activity against yeast cells of Hc. PM were also studied for comparative purposes and delineation of antifungal mechanisms. A previously devised system for culturing non-adherent non-ingested yeast cells as well as adherent-ingested yeast cells present in cocultures was used [14]. This permitted the analysis of extracellular, as well as

Correspondence: Dr Elmer Brummer, Division of Infectious Diseases, Department of Medicine, Santa Clara Valley Medical Centre, 751 South Bascom Avenue, San Jose, CA 95128, USA.

intracellular effects, on yeast cells cocultured with macrophages.

MATERIALS AND METHODS

Macrophages

Lungs of male BALB/c mice purchased from Simonsen Lab. (Gilroy, CA), 8–12 weeks old, were lavaged as previously described [15]. Lavaged cells were washed once, counted and suspended to $1{\cdot}5 \times 10^6$/ml complete tissue culture medium (CTCM). CTCM consisted of RPMI 1640, 10% fetal bovine serum (FBS; v/v), penicillin (100 U/ml) and streptomycin (100 μg/ml). BAM were selected by adherence by incubating lavaged cells (0·1 ml/microtest plate well) for 2 h at 37°C in 5% CO_2–95% air. Non-adherent cells were removed by washing the monolayers once with RPMI 1640. Of incubated cells, 90% were adherent ($1{\cdot}35 \times 10^6$ BAM/well) [16].

Resident PM were obtained from peritoneal cells as previously described [17]. Briefly, peritoneal cells ($2{\cdot}5 \times 10^6$/ml CTCM) were plated 0·1 ml per microtest plate well, incubated for 2 h at 37°C in 5% CO_2–95% air, and non-adherent cells removed by washing. Of plated cells, 50% were adherent, of which 90% were non-specific esterase-positive [17].

Treatment and challenge of macrophages

In most experiments monolayers were treated with 1000 U/ml IFN-γ, a gift from Genentech Inc. (South San Francisco, CA) plus 10 ng/ml LPS (Sigma Chemical Co., St Louis, MO) or CTCM for 48 h in 5% CO_2–95% air (reagent concentrations shown optimal in preliminary experiments). After culture supernatants were aspirated, macrophages were challenged with 0·1 ml of a yeast cell suspension of Hc (isolate G217B in CTCM + 10% fresh mouse serum) for 3 h in killing assays or 24 h in fungistatic assays. In 3-h killing assays the inoculum colony-forming units (CFU; 791 ± 126, $n = 5$) did not differ significantly from CFU of Hc in medium alone.

Histoplasma capsulatum

A well characterized isolate of *H. capsulatum*, G217B (ATCC 26032) was grown in CTCM at 37°C in 5% CO_2–95% air for 48–72 h. Yeast cells were washed once with saline and fungal units counted with a haemacytometer. The morphology of fungal units consisted of units with one ($28 \pm 6\%$), two ($34 \pm 9\%$), three ($22 \pm 4\%$), four ($12 \pm 4\%$), or five or more ($4 \pm 2\%$) cells ($n = 3$). Viability of fungal units was estimated using the fluorescein diacetate-ethidium bromide (FDA–EB) viability staining [18]. A fungal unit was determined to be viable if one or more cells in the unit stained positive.

Plating medium

The culture filtrate supplemented brain heart infusion (BHI) agar (from Difco, Detroit, MI) medium with good plating efficiency for Hc was used. A culture filtrate of isolate G217B was produced in glucose-cysteine broth as described by Kwon-Chung & Tewari [19]. Supplemented medium consisted of 445 ml BHI agar, 10 ml culture filtrate, and 50 ml of a 1% bovine serum albumin (BSA) solution. Preliminary experiments showed that enumeration of CFU of Hc grown in CTCM gave 100% correlation with viability of fungal units determined by the FDA–EB stain. Viability of fungal units assessed by FDA–EB staining was $81 \pm 3\%$ ($n = 5$) of haemacytometer counts.

Fungicidal and fungistatic activity

To measure fungicidal activity, quadruplicate cultures were harvested after 3 h of incubation at 37°C in 5% CO_2–95% air. Harvesting was done using distilled water to lyse macrophages as previously described [16]. Microscopic examination of microtest plate wells verified removal of macrophages. Appropriate dilution of harvested material was plated on supplemented BHI agar plates. Microscopic examination of harvested material showed that the morphology of fungal units (number of yeast cells per fungal unit) was similar to that of the inoculum, consequently reduction of inoculum CFU was not due to clumping. Fungicidal activity was calculated by the formula: $(1 - (\text{experimental CFU/inoculum CFU})) \times 100$.

Fungistatic activity was measured after incubation of quadruplicate cultures for 24 h at 37°C in 5% CO_2–95% air. Non-adherent non-ingested yeast cells were harvested by aspirating culture supernatants and rinsing the monolayer once with RPMI 1640. Non-adherent non-ingested CFU were determined by plating harvested material on supplemented BHI agar plates. Adherent-ingested yeast cells were harvested using distilled water to lyse macrophages and CFU were measured by plating harvested material on supplemented BHI agar plates. The combined non-adherent non-ingested and adherent-ingested CFU (total CFU per culture) was used to determine fungistatic activity. Fungistatic activity was calculated by the formula: $(1 - (\text{experimental coculture CFU/control coculture CFU})) \times 100$.

Fungicidal and fungistatic mechanisms

Superoxide dismutase (SOD) and catalase (CAT) from Sigma were used as scavengers of products from the oxidative burst, e.g. superoxide anion and hydrogen peroxide, respectively. NMMA (Sigma), a competitive inhibitor of L-arginine [20], was used at 0·2 mM. SOD and CAT were prepared and used at concentrations previously reported to be effective [21]. SOD, CAT or NMMA in medium had no significant effect on the viability or multiplication of *H. capsulatum* in 24-h assays compared with medium alone. Likewise, SOD, CAT or NMMA added to control macrophage cocultures had no significant effect on the number of CFU compared with CFU in cultures of macrophages with medium alone. Activity of CAT was negated by heating (125°C, 15 min).

L-arginine supplementation

In view of a recent report that L-arginine was rapidly depleted from culture medium by activated macrophages [22], we tested the effect of adding L-arginine to cocultures. Sets of cocultures were supplemented with 1 mM L-arginine at the beginning of the experiment (day 0) and again on days 1 and 2. Sets of cocultures without L-arginine supplementation served as controls. The fungistatic activity of the day 0 coculture set was assessed 24 h later, day 1 sets of cocultures after 48 h of challenge, and day 2 sets of cocultures after 72 h of incubation.

Statistical analysis

Comparison between groups was made by Student's t-test, with significance assumed to be $P < 0{\cdot}05$. Data are expressed as mean ± s.d.

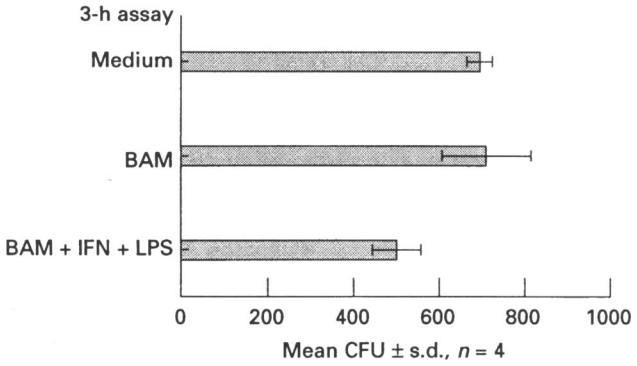

Fig. 1. Killing of *Histoplasma capsulatum* by activated bronchoalveolar macrophages (BAM). BAM activated with IFN plus lipopolysaccharide (LPS) for 48 h and control BAM cultured in medium were challenged with yeast cells for 3 h. Reduction of inoculum colony-forming units (CFU) by activated BAM compared with control BAM from a representative experiment is shown.

RESULTS

Fungicidal activity

Fungicidal activity of activated BAM for Hc is shown in Fig. 1. Control BAM failed to kill Hc, whereas BAM activated by IFN plus LPS significantly reduced inoculum CFU by 28%.

When non-adherent non-ingested CFU from control and activated BAM cocultures were compared (162 ± 22 *versus* 136 ± 21, $n = 4$) the differences were not significant. Reduction of CFU by activated BAM compared with control BAM was associated with the adherent-ingested CFU (366 ± 35 *versus* 547 ± 82). Similar results were found when non-adherent non-ingested CFU and adherent-ingested CFU from activated and control PM cocultures were compared. This indicates that killing was associated with the adherent-ingested yeast cell population in cocultures containing activated macrophages.

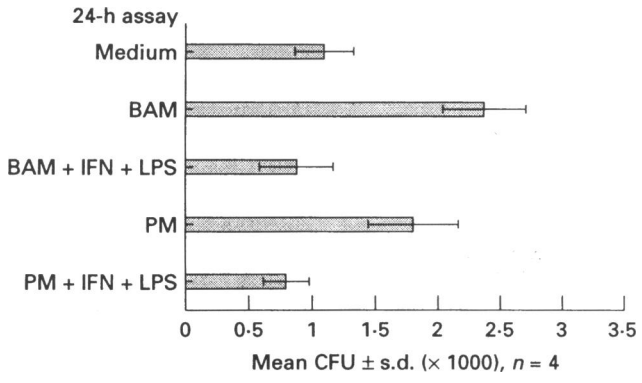

Fig. 2. Fungistasis of *Histoplasma capsulatum* by activated bronchoalveolar macrophages (BAM) and peritoneal macrophages (PM). BAM and PM were activated with IFN plus lipopolysaccharide (LPS) for 48 h. Control BAM and PM were cultured in medium alone for 48 h. Treated BAM and PM were cocultured with an inoculum of 760 ± 50 colony-forming units (CFU) of *H. capsulatum* for 24 h and CFU per culture determined. Inhibition of *H. capsulatum* multiplication in activated BAM and PM cocultures compared with multiplication in control BAM and PM cocultures from a representative experiment is shown.

When harvested material pelleted by centrifugation was examined microscopically the morphology of the fungal units, e.g. average number of yeast cells per fungal unit, was not significantly different from the inoculum ($2{\cdot}27 \pm 0{\cdot}19$). These observations indicate that clumping could not explain reduction of inoculum CFU.

Fungistatic activity

Fungistatic activity of BAM for Hc is shown in Fig. 2. Multiplication of yeast cells was inhibited (67%, $P < 0{\cdot}001$) in activated BAM cocultures compared with that in control BAM cocultures (880 ± 290 *versus* 2368 ± 328 CFU). Figure 2 shows that the advantage for the fungus of multiplication in a cellular milieu was neutralized when activated macrophages were present. Inhibition of multiplication in activated BAM cocultures compared with control BAM cocultures was associated with both non-adherent non-ingested CFU (activated 180 ± 58 *versus* control 360 ± 96, $P < 0{\cdot}02$) and adherent-ingested CFU (activated 700 ± 232 *versus* control 2008 ± 232, $P < 0{\cdot}001$). This suggests that some fungistasis was occurring extracellularly as well as in conjunction with adherent-ingested yeast cells and activated BAM.

In parallel experiments with PM results were similar to those found with BAM. Activated PM cocultures significantly ($P < 0{\cdot}02$) inhibited multiplication of yeast cells compared with control PM cocultures (Fig. 2). Inhibition of multiplication was found in both the non-adherent non-ingested and the adherent-ingested population of cocultures.

Fungistasis as measured by fewer CFU in activated BAM or PM cocultures compared with CFU in control BAM and PM cocultures was not due to clumping. Microscopic examination of material pelleted by centrifugation showed that fungal units had morphology similar to that of the inoculum.

Fungicidal mechanism(s)

Killing by activated BAM could be inhibited by catalase or NMMA, but not SOD (Table 1). Inhibition of killing by catalase suggests a role for hydrogen peroxide, a product of the oxidative burst, in the fungicidal mechanism. Non-significant enhancement of killing by activated BAM in the presence

Table 1. Fungicidal activity of bronchoalveolar macrophages (BAM) activated by IFN + lipopolysaccharide (LPS) for *Histoplasma capsulatum*

BAM*	Scavengers	Per cent killing	Experiments	*P*†
Medium	0	0 ± 0	4	–
IFN + LPS	0	25 ± 12	4	<0·01
IFN + LPS	SOD	32 ± 7	2	<0·01
IFN + LPS	CAT	6 ± 1	2	NS‡
IFN + LPS	NMMA	0 ± 0	2	NS

* Cultured for 48 h with medium or IFN + LPS before 3 h challenge with *H. capsulatum* plus medium, superoxide dismutase (SOD, 1000 U/ml), catalase (CAT, 20 000 U/ml), or N^G-monomethyl L-arginine (NMMA, 0·2 mM).

† Statistical significance compared with BAM cultured with medium (control BAM).

‡ NS, Not significant compared with control BAM ($P > 0{\cdot}05$).

Table 2. Fungicidal activity of peritoneal macrophages (PM) activated with IFN + lipopolysaccharide (LPS) for *Histoplasma capsulatum*

PM*	Scavengers	Per cent killing	Experiments	P†
Medium	0	8 ± 9	5	–
IFN + LPS	0	25 ± 8	5	<0·02
IFN + LPS	SOD	19 ± 6	2	NS‡
IFN + LPS	CAT	0 ± 0	2	NS
IFN + LPS	NMMA	0 ± 0	3	NS

* Cultured for 48 h with medium or IFN + LPS before 3 h challenge with *H. capsulatum* plus medium, superoxide dismutase (SOD, 1000 U/ml), catalase (CAT, 20 000 U/ml), or N^G-monomethyl-L-arginine (NMMA, 0·2 mM).

† Statistical significance compared with PM cultured with medium (control PM).

‡ NS, Not significant compared with control PM ($P > 0·05$).

Table 3. Fungistatic activity of peritoneal macrophages (PM) activated with IFN + lipopolysaccharide (LPS) for *Histoplasma capsulatum*

PM*	Scavengers	Per cent fungistasis	Experiments	P†
Medium	0	0 ± 0	4	–
IFN + LPS	0	57 ± 5	4	<0·001
IFN + LPS	SOD	100 ± 0	3	<0·001
IFN + LPS	CAT	55 ± 6	3	<0·001
IFN + LPS	NMMA	0 ± 0	3	NS‡

* Cultured for 48 h with medium or IFN + LPS before challenge with *H. capsulatum* for 24 h in medium, or medium plus superoxide dismutase (SOD, 1000 U/ml), catalase (CAT, 20 000 U/ml), or N^G-monomethyl-L-arginine (NMMA, 0·2 mM).

† Statistical significance compared with PM cultured with medium (control PM).

‡ NS, Not significant compared with control PM ($P > 0·05$).

of SOD, which in addition to dismutation of superoxide also promotes the generation of hydrogen peroxide, is consistent with a role for hydrogen peroxide in the fungicidal mechanism (Table 1).

NMMA was the most effective inhibitor of killing by activated BAM. This indicates that nitric oxide synthase (NOS) was induced in activated BAM compared with control BAM, and fungicidal products of the L-arginine NOS pathway were generated early in the interaction of activated BAM and Hc. The enhancement of killing by SOD could also occur by removal of superoxide anion, as the latter may directly decrease levels of nitric oxide [23].

Killing by activated PM could also be inhibited by catalase or NMMA, but not SOD (Table 2). These results show that the killing mechanism is similar in both activated BAM and PM.

Fungistatic mechanism(s)

The fungistatic activity of activated BAM, 61 ± 4%, $n = 3$, could be inhibited by NMMA, but not by catalase or SOD (data not shown). Similarly, fungistatic activity of activated PM could only be inhibited by NMMA, as shown by a representative experiment (Fig. 3). Data from three experiments are given in Table 3. SOD significantly ($P < 0·001$) enhanced fungistasis by activated PM (Table 3). Since SOD did not have this effect on control PM (data not shown), this phenomenon was associated with activated PM.

Effect of L-arginine supplementation

L-arginine supplementation of control cocultures for 24 h did not significantly affect multiplication of yeast cells compared with non-supplemented control cocultures (bars 1 and 3, 24 h; Fig. 4). The fungistatic activity of 24 h L-arginine supplementation of activated PM cocultures did not significantly differ from that of non-supplemented activated PM cocultures (68% and 78%, respectively; bars 2 and 4, 24 h, Fig. 4).

Supplementation of control PM cocultures with L-arginine for 2 days in a row enhanced multiplication of Hc ($P < 0·02$)

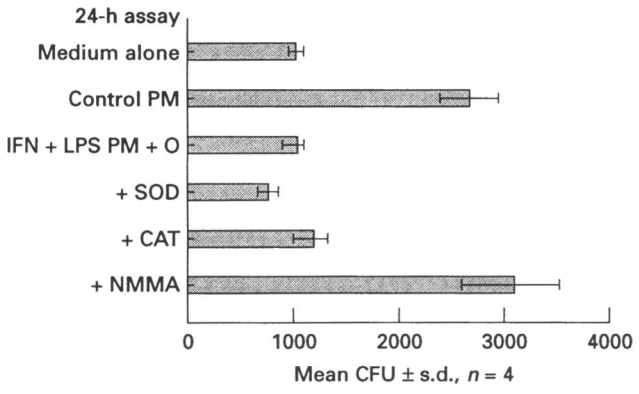

Fig. 3. Inhibition of fungistasis by N^G-monomethyl-L-arginine (NMMA). Multiplication of 973 ± 80 colony-forming unit (CFU) inoculum of *Histoplasma capsulatum* in IFN plus lipopolysaccharide (LPS)-activated peritoneal macrophage (PM) cocultures compared with that in control PM cocultures over 24 h is depicted. Failure of catalase (CAT, 20 000 U/ml) or superoxide dismutase (SOD, 1000 U/ml) to inhibit fungistatic activity of activated PM is shown. NMMA (0·2 mM) is shown to abrogate fungistatic activity of activated PM.

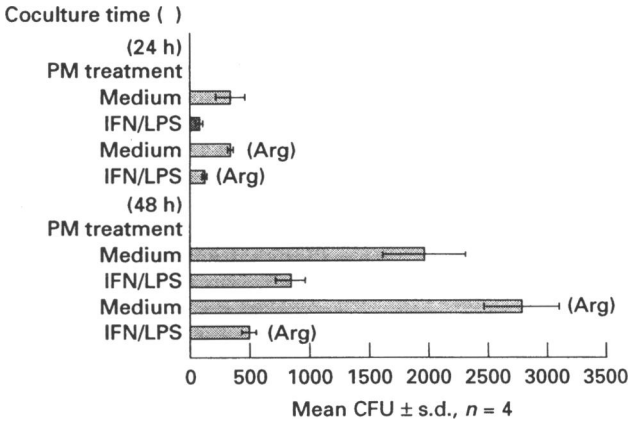

Fig. 4. Effect of supplemental L-arginine. Fungistatic activity of IFN plus lipopolysaccharide (LPS)-activated peritoneal macrophages (PM), without or with 1 mM L-arginine supplementation, after 24 h and 48 h of coculture with an inoculum of 340 ± 50 colony-forming units (CFU) of *Histoplasma capsulatum* is shown. Arg, Supplemental L-arginine.

compared with non-supplemented control cocultures when assessed 48 h after challenge (bars 1 and 3, 48 h, Fig. 4). However, activated PM cocultures supplemented with L-arginine for 2 days in a row significantly ($P < 0{\cdot}01$) enhanced fungistatic activity (75%) compared with non-supplemented activated PM cocultures (54%) (bars 2 and 4, 48 h, Fig. 4).

After 3 days of supplementation and incubation the number of CFU increased to high levels in non-activated PM cocultures, e.g. $37\,680 \pm 3900$ CFU (without L-arginine supplementation). The growth in non-activated PM cocultures supplemented with L-arginine was significantly less ($P < 0{\cdot}01$), i.e. $26\,980 \pm 2220$ CFU. These results indicate that PM cocultures incubated for 3 days with CTCM and supplemental L-arginine can restrict growth compared with non-supplemented cocultures. It may be possible that some induction of NO synthase takes place during prolonged coculture, and supplemental L-arginine helps restrict growth.

The growth in activated PM cocultures, 8060 ± 1550 CFU (non-supplemented cocultures), was significantly more ($P < 0{\cdot}05$) than in L-arginine-supplemented cocultures (5660 ± 768 CFU; data not shown). This shows that activated PM in 3-day cultures are more effective in restricting growth when supplemented with L-arginine.

DISCUSSION

We report here that BAM or PM activated *in vitro* are fungicidal for yeast cells of Hc. To our knowledge this is the first demonstration of Hc killing by activated murine BAM. This was made possible by using a culture methodology that measured CFU of Hc. Microscopic staining methods used in previous reports could not detect killing in a short-term assay [10].

Activated BAM when compared with control BAM inhibited the multiplication of Hc in 24-h cocultures. Inhibition was largely associated with the ingested-adherent yeast cell population in cocultures. This is consistent with the previously reported inhibition of intracellular multiplication of Hc by activated PM using the macrophage monolayer staining method [8,10].

The culture method used in the present work allowed comparison of *H. capsulatum* multiplication in medium alone with that in control or activated BAM cocultures. Multiplication of *H. capsulatum* in activated BAM cocultures was inhibited compared with control BAM cocultures, but not when compared with multiplication in medium alone. This indicates that yeast cells in the control BAM cocultures, especially the ingested-adherent yeast cells, have a nutritional advantage. The enhanced multiplication of ingested-adherent yeast cells in control BAM cocultures can be accounted for by the macrophage transferrin receptor–transferrin transport system, by which labile iron is made available intracellularly for Hc [24]. Activated macrophages have down-regulated transferrin receptors [25] and growth of intracellular pathogens can be inhibited under these conditions by iron deprivation [13,24]. The low rate of multiplication of yeast cells in tissue culture medium, where the only source of iron is serum transferrin, suggests the difficulty that Hc has in obtaining labile iron under these conditions.

The fungicidal mechanism of activated macrophages for Hc depends on presence of hydrogen peroxide and the activity of the L-arginine nitric oxide synthase pathway. Activated macrophages have an enhanced oxidative burst in response to various fungal stimuli [21] including opsonized Hc [26]. Consequently, it is not surprising that hydrogen peroxide is present in short-term killing assays and plays a role in the fungicidal mechanism of activated BAM or PM. It is not clear at this time whether the role of hydrogen peroxide in killing is indirect, e.g. interaction with nitric oxide [23], or direct.

Products of the L-arginine nitric oxide synthase pathway that are involved in killing *H. capsulatum* are difficult to identify [27]. The chemistry of nitric oxide is rapidly evolving, and there is now good evidence that nitric oxide reacting with reactive oxygen radicals [23] produces peroxynitrites which nitrates tyrosine residues of proteins [28,29]. The precise damage that products of such reactions may have on killing Hc remains to be determined.

Catalase inhibited killing in short-term assays but could not inhibit fungistasis in long-term 24-h assays. This suggests that hydrogen peroxide, generated by an initial oxidative burst, plays only an early transient role in the antifungal mechanism.

NMMA completely abrogated the fungistatic activity of activated macrophages for Hc. In contrast, catalase had no effect. On the other hand, SOD enhanced the fungistasis of activated macrophages. This may be related to the effect of superoxide anion in reducing levels of nitric oxide [23], as mentioned previously. How the products of the ongoing L-arginine NOS pathway fit in with the iron deprivation mechanism for fungistasis [24] is not clear at this time. This issue awaits further investigation.

Vodovotz *et al.* [22] have reported that activated macrophages rapidly deplete L-arginine from culture medium. The depletion of L-arginine exceeded that of nitrate production, indicating that macrophage arginase was involved as well as the NOS pathway. Daily addition of L-arginine (2 mM) to cultures more than doubled the production of nitrate. Here we report that, under the experimental conditions used, addition of L-arginine (1 mM) for 2 days significantly increased the fungistatic activity of activated macrophages compared with controls.

It is not known how the limitations of L-arginine *in vitro* relate to the fungistatic activity of activated macrophages *in vivo*. *In vivo* activated macrophages could be resupplied with nutrients which are being depleted, e.g. L-arginine, and also be influenced by the presence of IFN and other cytokines.

In conclusion, activated BAM and PM can kill yeast cells of Hc, and the mechanism(s) depend on hydrogen peroxide and products of the NOS pathway. Fungistatic activity of activated macrophages for Hc requires products of the NOS pathway and is influenced by the availability of L-arginine.

REFERENCES

1 De Monbreun WA. The cultivation and cultural characteristics of Darling's *Histopasma capsulatum*. Am J Trop Med 1934; **14**:93–125.

2 Schwarz J. The pathogenesis of histoplasmosis. Trans NY Acad Sci 1958; **20**:541–8.

3 Graybill JR. Histoplasmosis and AIDS. J Infect Dis 1988; **158**:623–6.

4 Salzman SH, Smith RL, Aranda CP. Histoplasmosis in patients at risk for acquired immunodeficiency syndrome in a nonendemic setting. Chest 1988; **93**:916–21.

5 Kurita N, Brummer E, Yoshida S, Nishimura K, Miyaji M. Antifungal activity of murine polymorphonuclear neutrophils against *Histoplasma capsulatum*. J Med Vet Mycol 1991; **29**:133–43.
6 Brummer E, Kurita N, Yoshida S, Nishimura K, Miyaji M. Fungistatic activity of human neutrophils against *Histoplasma capsulatum*: correlation with phagocytosis. J Infect Dis 1991; **164**:158–62.
7 Newman SL, Gootee L, Gabay J. Human neutrophil-mediated fungistasis against *Histoplasma capsulatum*. Localization of fungistatic activity to the azurphil granules. J Clin Invest 1993; **92**:624–31.
8 Kimberlin CL, Hariri AR, Hempel HO, Goodman NL. Interaction between *Histoplasma capsulatum* and macrophages from normal and treated mice: comparison of mycelial and yeast phases in alveolar and peritoneal macrophages. Infect Immun 1981; **34**:6–10.
9 Howard DH. Intracellular growth of *Histoplasma capsulatum*. J Bacteriol 1965; **89**:518–23.
10 Wu-Hsieh BA, Howard DH. Inhibition of the intracellular growth of *Histoplasma capsulatum* by recombinant murine gamma-interferon. Infect Immun 1987; **55**:1014–6.
11 Brummer E, Stevens DA. Effect of macrophage colony-stimulating factor on macrophage morphology, phagocytosis and intracellular multiplication of *Histoplasma capsulatum*. Int J Immunopharmacol 1994; **16**:171–6.
12 Prochnow JJ, Page MI, Loosli CJ. Early pathogenesis of experimental histoplasmosis. Arch Pathol 1960; **69**:413–26.
13 Cano LE, Gomez B, Brummer E, Restrepo A, Stevens DA. Inhibitory effect of deferoxamine or macrophage activation on transformation of *Paracoccidioides brasiliensis* conidia ingested by macrophages: reversal by holotransferrin. Infect Immun 1994; **62**:1494–6.
14 Brummer E, Kurita N, Yoshida S, Nishimura K, Miyaji M. Killing of *Histoplasma capsulatum* by gamma-interferon activated human monocyte-derived macrophages: evidence for a superoxide anion-dependent mechanism. J Med Microbiol 1991; **35**:29–34.
15 Sugar AM, Brummer E, Stevens DA. Murine pulmonary macrophages: evaluation of lung lavage fluids, miniaturized monolayers, and candidacidal activity. Am Rev Respir Dis 1983; **127**:110–22.
16 Brummer E, Stevens DA. Activation of pulmonary macrophages for fungicidal activity by gamma-interferon or lymphokines. Clin Exp Immunol 1987; **70**:520–8.
17 Brummer E, Morrison CJ, Stevens DA. Recombinant and natural gamma-interferon activation of macrophages *in vitro*: different dose requirements for induction of killing activity against phagocytizable and non-phagocytizable fungi. Infect Immun 1985; **49**:724–30.
18 Calich VLG, Purchio A, Paula A. A new fluorescent viability test for fungi cells. Mycopathologia 1978; **66**:175–7.
19 Kwon-Chung KJ, Tewari RP. Determination of viability of *Histoplasma capsulatum* yeast cells grown *in vitro*: comparison between dye and colony count methods. J Med Vet Mycol 1987; **25**:107–14.
20 Granger DL, Hibbs JB, Perfect JR, Durack DT. Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. J Clin Invest 1988; **81**:1129–36.
21 Brummer E, Stevens DA. Candidacidal mechanisms of peritoneal macrophages activated with lymphokines or gamma-interferon. J Med Microbiol 1989; **28**:173–81.
22 Vodovotz Y, Kwon NA, Popischil M, Manning J, Paik J, Nathan C. Inactivation of nitric oxide synthase after prolonged incubation of mouse macrophages with interferon-gamma and bacterial lipopolysaccharide. J Immunol 1994; **152**:4110–8.
23 Lane TE, Wu-Hsieh BA, Howard DH. Iron limitation and the gamma-interferon mediated antihistoplasma state of murine macrophages. Infect Immun 1991; **59**:2274–8.
24 Hamilton TA, Gray PW, Adams DO. Expression of transferrin receptor on murine peritoneal macrophages is modulated by *in vitro* treatment with interferon-gamma. Cell Immunol 1984; **89**:478–88.
25 Wolf JE, Kerchgerger V, Kobayashi GS, Little JR. Modulation of the macrophage oxidative burst by *Histoplasma capsulatum*. J Immunol 1987; **138**:582–6.
26 Green SJ, Nacy CA, Meltzer MS. Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to Leishmania and other intracellular pathogens. J Leuk Biol 1991; **50**:93–103.
27 Nakamura LT, Wu-Hsieh BA, Howard DH. Recombinant murine gamma-interferon stimulates macrophages of the RAW cell line to inhibit intracellular growth of *Histoplasma capsulatum*. Infect Immun 1994; **62**:680–4.
28 Ye Y, Anderson P, Beckman JS. Nitration of protein tyrosine residues in human atherosclerosis. FASEB J 1994; **8**:594.
29 Ye Y, Galliani CA, Beckman JS, Matalon S. Detection of nitrotyrosine in patients with the adult respiratory distress syndrome. FASEB J 1994; **8**:896.