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Enhancing effect of oxygen radical scavengers on murine macrophage anticryptococcal activity through production of nitric oxide

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SUMMARY

We examined the roles of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) in interferon-gamma (IFN-γ)-induced cryptococcostatic activity of murine peritoneal macrophages using N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of RNI synthesis, and superoxide dismutase (SOD) and catalase, oxygen radical scavengers. IFN-γ-activated macrophages produced nitric oxide (NO) in a dose-dependent manner, as measured by increased nitrite concentration in the culture supernatant. IFN-γ also enhanced the suppressive effect on cryptococcal growth in a similar dose-dependent manner. The induction of killing activity and NO production by an optimal dose of IFN-γ (100 U/ml) was virtually suppressed by 500 μM L-NMMA. These results confirmed the importance of the RNI-mediated effector mechanism in anticryptococcal activity of macrophages. SOD and catalase significantly enhanced the cryptococcostatic activity of macrophages induced by a suboptimal dose of IFN-γ (20 U/ml). The augmenting effect of these reagents was mediated by NO, since they potentiated the production of NO by macrophages and their effects were totally blocked by L-NMMA. Our results indicate that the IFN-γ-induced anticryptococcal activity of macrophages is dependent mostly on RNI, and suggest that the ROI system down-regulates the effector mechanism for cryptococcostasis by suppressing the RNI system.

Keywords *Cryptococcus neoformans* macrophages interferon-gamma nitric oxide superoxide oxygen radical scavengers

INTRODUCTION

Reactive oxygen intermediates (ROI) have long been investigated as the major macrophage killing mechanism used against certain microbial parasites since the early report by Walker *et al.* on ROI-mediated killing of *Mycobacterium microti* by immunologically activated macrophages [1]. Recently, Chan *et al.* [2] demonstrated that the superoxide dismutase (SOD)- and catalase-sensitive killing mechanism did not play a critical role in the microbicidal activity of macrophages against *Myco. tuberculosis*. They further demonstrated that the ROI-deficient mutant macrophage kills the bacilli as effectively as its parent cell line. Thus, the role of ROI as the effector molecule in antimicrobial activity is controversial. Other mechanisms, including oxygen-independent killing [3,4] and a cytotoxic action mediated by reactive nitrogen intermediates (RNI) [5], have also been reported. The latter mechanism participates in the antimicrobial capability of macrophages against various pathogens [6–12]. For effective release of this inorganic mediator, activation of macrophages is necessary [13]. Interferon-gamma (IFN-γ) is a potent activator of macrophages [13] that is also known to enhance the killing activity of these cells [14–18].

Cryptococcus neoformans causes life-threatening disease, particularly in patients with impaired T cell function, such as in patients with AIDS [19], since host resistance against this pathogen is dependent on cell-mediated immunity [20]. IFN-γ plays a central role in the induction of anticryptococcal macrophage activity [15]. Furthermore, RNI is also an important effector molecule in the killing activity of macrophages [8]. However, it is unclear whether ROI-mediated killing is involved in IFN-γ-induced anticryptococcal macrophage activity.

In the present study, we examined the effect of N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of nitric oxide (NO) synthesis, or SOD and catalase, oxygen radical scavengers, on the cryptococcostatic activity of murine peritoneal macrophages induced by IFN-γ.

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MATERIALS AND METHODS

Animals

Female (BALB/c × DBA/2)F_1 mice were purchased from SLC Japan (Hamamatsu, Japan) and used in the present experiment at the age of 7–10 weeks. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our university. The anticryptococcal activity of macrophages derived from this strain of mice is not detected without stimulation, and is markedly induced by IFN-γ [21]. All mice were housed in a pathogen-free environment and received sterilized food and water at the Laboratory Animal Centre for Biomedical Science in University of the Ryukyus.

Cryptococcus neoformans

A serotype A-encapsulated strain of *C. neoformans*, YC-11, was obtained from a patient with pulmonary cryptococcosis. This strain killed a group of mice within 4 6 weeks after intratracheal instillation of 1×10^5 yeast cells [15]. The yeast cells were cultured at 31°C on a potato-dextrose agar (PDA) plate. The cells were collected after 4 or 5 days culture, washed three times in normal saline, and counted using a haemacytometer.

Culture medium and reagents

RPMI 1640 medium (GIBCO BRL, Grand Island, NY), fetal calf serum (FCS; Whitaker, Walkersville, MD), L-NMMA (Calbiochem Novabiochem, Tokyo, Japan), and SOD and catalase (Wako Chemical Co., Osaka, Japan) were purchased from the respective manufacturers. Murine recombinant IFN-γ was a generous gift from Daiichi Pharmaceutical Co. (Tokyo, Japan).

Preparation of peritoneal macrophages

Mice were injected intraperitonealy with 1·5 ml 3% thioglycolate. Peritoneal exudate cells were harvested 4 days later by two injection cycles of 5 ml cold RPMI 1640 supplemented with 10 mM HEPES. The obtained cells were cultured at $1{\cdot}5–2{\cdot}0 \times 10^6$/ml in glass dishes precoated with FCS, for 1 h in a 5% CO_2 incubator. Adherent cells were collected by dislodging with a rubber policeman after removal of non-adherent cells.

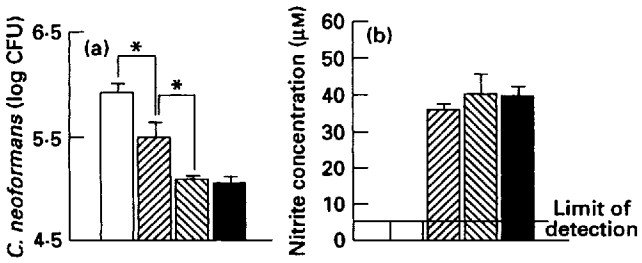

Fig. 1. Correlation of cryptococcostatic activity and nitric oxide (NO) production by macrophages. Macrophages were incubated at 1×10^6/ml with or without various concentrations of IFN-γ for 24 h. *Cryptococcus neoformans* was then added at 1×10^5/ml to each well and cultured for another 24 h. All pathogens were harvested and inoculated on potato-dextrose agar (PDA) culture plates after appropriate dilution with distilled water. The culture supernatant in each well was collected and assayed for its nitrite content. (a) Colony count. (b) Nitrite concentration. $^*P < 0{\cdot}05$. □, Control; ▨, IFN-γ 20 U/ml; ▧, IFN-γ 50 U/ml; ■, IFN-γ 100 U/ml.

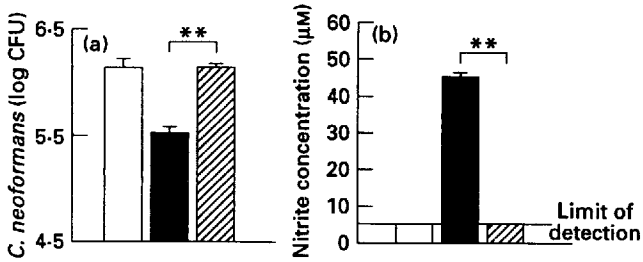

Fig. 2. Effect of N^G-monomethyl-L-arginine (L-NMMA) on cryptococcostatic activity and nitric oxide (NO) production by macrophages. Macrophages were incubated at 1×10^6/ml with 100 U/ml IFN-γ for 24 h in the presence or absence of 500 μM of L-NMMA. *Cryptococcus neoformans* was then added at 1×10^5/ml to each well and cultured for another 24 h. All pathogens were harvested and inoculated on potato-dextrose agar (PDA) culture plates after appropriate dilution with distilled water. The culture supernatant in well was collected and assayed for nitrite content. (a) Colony count. (b) Nitrite concentration. $^{**}P < 0{\cdot}01$. □, Control; ■, IFN-γ; ▨, IFN-γ + L-NMMA 500 μM.

Assessment of in vitro *fungistatic activity of macrophages*

Macrophages (1×10^6/well) were precultured with various amounts of IFN-γ, followed by addition of *C. neoformans* (1×10^5/well) 24 h later. After another 24 h of culture, the supernatant in each well was harvested, and each well received three cycles of washing with 1 ml of distilled water to destroy macrophages. *Cryptococcus neoformans* were harvested and adjusted to 10 ml with distilled water, diluted with sterile water, and inoculated on a PDA plate. The number of colonies was counted 2 days later.

NO assay

To estimate synthesis of NO by macrophages, the concentration of nitrite, a metabolite of NO, accumulating in the culture was measured using the method described by Stuehr & Nathan [22]. Briefly, 100 μl of supernatant were mixed with the same volume of Griess reagent and absorbance was read at 550 nm using an automated microplate reader. The concentration of nitrite was calculated from a standard curve.

Statistical analysis

Values are reported as mean ± s.d. The unpaired Student's *t*-test was used to compare differences between groups. $P < 0{\cdot}05$ was considered significant.

RESULTS

Correlation between cryptococcostatic activity and production of NO by IFN-γ-stimulated macrophages

Cryptococcostatic activity of peritoneal macrophages pretreated with IFN-γ for 24 h was examined following 24 h culture with *C. neoformans*. In the absence of IFN-γ, macrophages showed a marginal inhibition of the growth of yeast cells compared with that of culture medium without macrophages (data not shown). Treatment with IFN-γ markedly enhanced the fungistatic activity of macrophages. Higher doses of IFN-γ enhanced the growth inhibitory effects of

macrophages, with the maximum effect observed at IFN-γ concentrations > 50 U/ml (Fig. 1a).

Untreated macrophages did not produce any detectable amount of NO, while a considerable amount of nitrite was detected in the culture supernatant of macrophages stimulated with IFN-γ (Fig. 1b), similar to that in its effect on their cryptococcostatic activity.

Role of RNI in IFN-γ-induced cryptococcostatic macrophage activity

To define the role of RNI in IFN-γ-induced cryptococcostatic macrophage activity, the effect of L-NMMA, a competitive inhibitor of NO production, was examined in the next series of experiments. As shown in Fig. 2a, b, both cryptococcostatic activity and NO production of macrophages induced by 100 U/ml IFN-γ were completely suppressed by the addition of L-NMMA at 500 μM. The effect of L-NMMA was not due to a direct cytotoxic effect on macrophages, since the addition of 5 mM L-arginine completely blocked the effect (data not shown).

Role of ROI in IFN-γ-induced fungistatic activity of macrophages

To define the role of ROI in IFN-γ-induced cryptococcostatic macrophage activity, the effects of SOD and catalase were examined separately. The addition of these reagents significantly enhanced the cryptococcostatic activity of macrophages induced by a suboptimal dose of IFN-γ (20 U/ml) in a dose-dependent manner (Fig. 3a, b). In addition, these cryptococcostatic activities were closely related to NO production by macrophages (data not shown). When used alone without IFN-γ, SOD failed to influence the growth of *C. neoformans*, while catalase showed a small inhibitory effect on it (Fig. 3a, b).

Induction of NO production by SOD and catalase

To define the mechanism of action of SOD and catalase, the ability of these oxygen radical scavengers to induce NO synthesis by macrophages was examined in the presence or absence of suboptimal doses of IFN-γ. As shown in Fig. 4, both

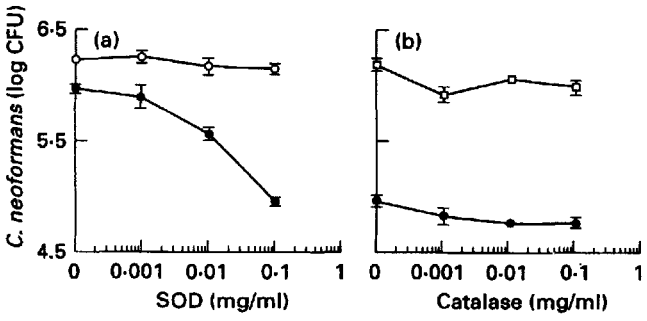

Fig. 3. Effect of oxygen radical scavengers on cryptococcostatic activity of macrophages. Macrophages were incubated at 1×10^6/ml for 24 h with or without 20 U/ml IFN-γ. Superoxide dismutase (SOD) (a) or catalase (b) was added at indicated concentrations 4 h before infection. *Cryptococcus neoformans* was then added at 1×10^5/ml to each well and cultured for another 24 h. All pathogens were harvested and inoculated on potato-dextrose agar (PDA) culture plates after appropriate dilution with distilled water. ○, Control; ●, with IFN-γ.

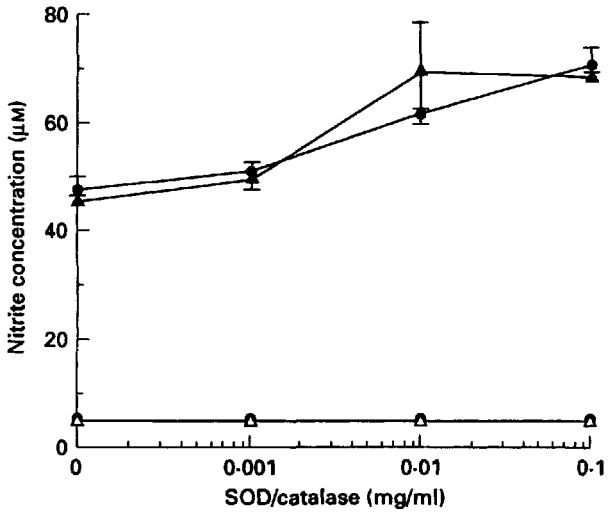

Fig. 4. Capability of oxygen radical scavengers to induce the production of nitric oxide (NO) by macrophages. Macrophages (1×10^6/ml) were cultured with or without 20 U/ml IFN-γ in the presence or absence of various doses of superoxide dismutase (SOD) or catalase for 48 h. The supernatants were collected and measured for nitrite content. ○, SOD; ●, IFN-γ + SOD; △, catalase; ▲, IFN-γ + catalase.

reagents significantly augmented NO production by macrophages stimulated with 20 U/ml of IFN-γ in a dose-dependent manner, with an optimal dose of 0·1 mg/ml for SOD and 0·01–0·1 mg/ml for catalase.

The stimulatory effects of SOD and catalase may have been produced by lipopolysaccharide (LPS) contaminating solutions used in the experiment, since LPS has been reported to have a similar effect on NO production [13]. The level of LPS in SOD and catalase used in the experiment was in both 300 pg/mg protein. Therefore, the next series of experiments examined whether LPS enhanced NO production by macrophages stimulated with 20 U/ml IFN-γ. LPS was used at a concentration (0·3–30 pg/ml) equivalent to that contaminating SOD and catalase solutions (0·001–0·1 mg/ml). LPS did not enhance NO production by IFN-γ-stimulated macrophages at all concentrations examined, and 30 pg/ml LPS did not influence the cryptococcostatic activity of IFN-γ-primed macrophages (data not shown).

Involvement of NO synthesis in the enhancing effects of SOD and catalase on the cryptococcostatic activity of macrophages

The above results suggested that the effects of SOD and catalase were probably mediated by enhanced production of NO. To confirm this possibility, the effect of a competitive inhibitor of NO synthesis was examined. As shown in Fig. 5, 500 μM L-NMMA completely abolished NO production and cryptococcostatic macrophage activity augmented by SOD and catalase.

DISCUSSION

In the present study, the roles of RNI and ROI in anti-cryptococcal activity of murine peritoneal macrophages stimulated with IFN-γ were examined using inhibitors against each mediator. Granger *et al.* demonstrated that L-arginine was

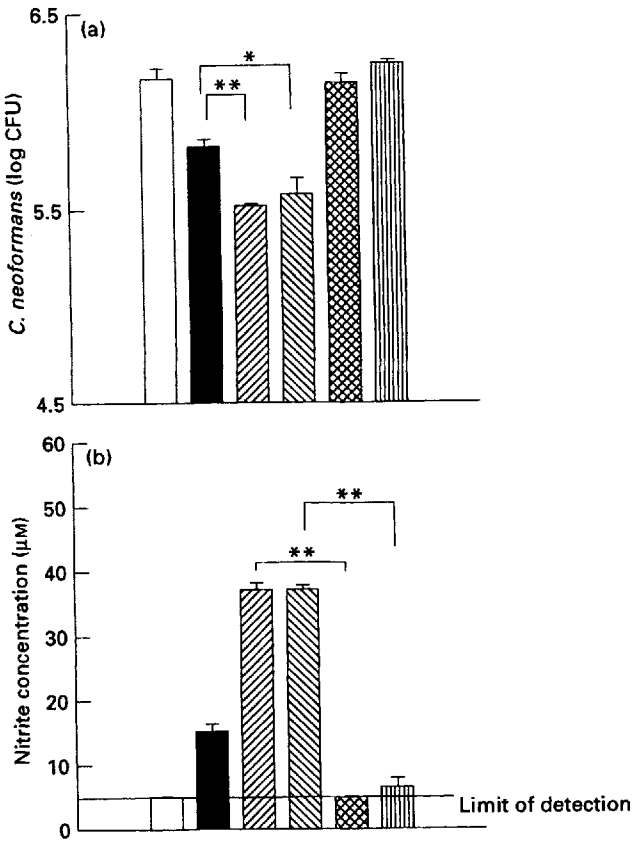

Fig. 5. Blocking effect of N^G-monoethyl-L-arginine (L-NMMA) on the enhancement of cryptococcostatic activity by oxygen radical scavengers. Macrophages were incubated at 1×10^6/ml with or without 20 U/ml IFN-γ for 24 h in the presence or absence of L-NMMA (500 μM). Superoxide dismutase (SOD) or catalase was added at indicated concentrations 4 h before the addition of yeast cells. *Cryptococcus neoformans* was then added at 1×10^5/ml to each well and cultured for another 24 h. All pathogens were harvested and inoculated on potato-dextrose agar (PDA) culture plates after appropriate dilution with distilled water, and the supernatants were measured for nitrite content. (a) Colony count. (b) Nitrite concentration. $^*P < 0.05$; $^{**}P < 0.01$. □, Control; ■, IFN-γ: ▨, IFN-γ + SOD; ▧ IFN-γ + catalase; ▩, IFN-γ + SOD+ L-NMMA; ▤, IFN-γ + catalase+ L-NMMA.

required for macrophage fungistatic activity, and that this activity was mediated by RNI [8]. Our results confirm and extend these early findings demonstrating that IFN-γ-induced cryptococcostatic macrophage activity was closely related to NO synthesis and strongly blocked by its competitive inhibitor, L-NMMA. Our results also support the recent finding of Alspaugh & Granger [23] demonstrating that *C. neoformans* is susceptible to the killing activity of NO synthesized *in vitro* by sodium nitrite under acidic conditions. Thus, these results confirm the significant role of this inorganic mediator in suppressing the growth of *C. neoformans*.

The exact role of ROI in the anticryptococcal activity of macrophages is not fully understood. The presence of melanin in *C. neoformans* is reported to protect the fungus from killing by oxygen metabolites [24]. The respiratory burst and related events, that result in the production of O_2^-, H_2O_2, $^{\cdot}HO$, and $^1O_2$, operate by enhancing bacterial killing by neutrophils [25]. However, the significance of this system in assisting the macrophages in killing facultative intracellular pathogens has not yet been established. Furthermore, the activity of myeloperoxidase (MPO) is lower in mature macrophages compared with neutrophils. This enzyme acts on H_2O_2 with chloride to synthesize hypochlorous acid and $^1O_2$ [26]. Earlier studies reported oxygen-independent microbiostatic activity of macrophages against several pathogens, such as *Toxoplasma gondii* [3], *Chlamydia psittaci* [4], *Myco. bovis* BCG [27], *Leishmania donovani* [28], and *Schistosoma mansoni* [29]. The findings were supported by a recent study of Chan *et al.* [2] demonstrating that ROI was not a principal mediator in the antimycobacterial capability of activated macrophages. In their study, the killing activity of ROI-deficient mutant macrophages against *Myco. tuberculosis* was equivalent to that of ROI-generating parent macrophages. Furthermore, these workers also demonstrated that the killing activity was not inhibited by oxygen radical scavengers. The present study extends these early findings by demonstrating that IFN-γ-induced anticryptococcal activity of macrophages is resistant to oxygen radical scavengers. Considered together, these results indicate that RNI, but not the ROI system, play a critical role in suppressing the growth of *C. neoformans*.

The strain of *C. neoformans* used in the present study was highly virulent, as shown by its ability to kill mice infected with small numbers of the yeast cells as quickly as 4 or 6 weeks after infection [15]. Since melanin is known as one of the virulence factors [30], this strain may contain more melanin and exert higher antioxidant activity than other less virulent strains. At the moment, the effect of oxygen radical scavengers on macrophage killing activity against less virulent strains remains unexamined. Thus, further studies using various strains of this microorganism are necessary to define critically the role of ROI in the anticryptococcal activity of macrophages.

Several investigators suggested that the respiratory burst interacts with the L-arginine-dependent metabolic system in neutrophils an other inflammatory cells [31,32]. According to our results, oxygen radicals may be suppressive to the RNI system, since scavengers of these radicals enhanced NO synthesis and anticryptococcal activity of IFN-γ-activated macrophages. Previous studies demonstrated that in the presence of O_2^-, NO is converted to peroxynitrite anion [33]. Furthermore, O_2^- is also involved in the breakdown of endothelium-derived vascular relaxing factor [34]. Therefore, SOD may prolong the half-life of NO by scavenging O_2^-, increase the production of NO and augment the cryptococcostatic activity of macrophages. However, alternative mechanisms may include a direct cytotoxic effect of peroxynitrite against certain microorganisms [35,36]. In this regard, a recent study by Clancy *et al.* [37] demonstrated that NO directly interferes with the enzymatic activity of NADPH oxidase to synthesize O_2^-. The precise effect of O_2^- on the activity of NO synthase (NOS) has not, however, been elucidated. This approach may allow a better understanding of the mechanisms activating L-arginine-dependent metabolic pathways by oxygen radical scavengers.

NOS is induced by various stimulators such as LPS, IFN-γ, tumour necrosis factor-alpha (TNF-α), and IL-1 [13,38]. LPS alone has little effect, but it markedly augments NOS production in macrophages primed with IFN-γ [13,38]. Therefore, contamination with LPS in the present study could possibly

have influenced the result of SOD and catalase. However, our study excluded such a possibility by demonstrating a lack of effect by LPS on NO production and fungistatic activity by IFN-γ-primed macrophages.

In conclusion, the significance of the two major killing systems, ROI and RNI, is thought to be distinct between host defence mechanisms against infection caused by different microorganisms. In cryptococcal infection, the L-arginine-dependent metabolic pathway plays a central role, and may be regulated by the respiratory burst and related events.

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