Peroxynitrite Contributes to the Candidacidal Activity of Nitric Oxide-Producing Macrophages

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Nitric oxide (NO) is associated with functions as diverse as peristalsis, blood flow, neuroendocrine secretion, visual transduction, smooth muscle relaxation, and microbial killing (H. H. W. H. Schmidt and V. Walter, *Cell* 78:919–925, 1994). Despite the well-established role of NO in macrophage candidacidal activity (E. Cenci, L. Romani, A. Mancacci, R. Spaccapelo, E. Schiaffella, P. Puccetti, and F. Bistoni, *Eur. J. Immunol.* 23:1034–1038, 1993; J. Jones-Carson, A. Vazquez-Torres, H. Van der Heide, R. D. Wagner, T. Warner, and E. Balish, *Nature Med.* 1:552–557, 1995; and A. Vazquez-Torres, J. Jones-Carson, T. Warner, and E. Balish, *J. Infect. Dis.* 172:192–198, 1995), NO is not directly candidacidal for *Candida albicans* (A. Vazquez-Torres, J. Jones-Carson, and E. Balish, *Infect. Immun.* 63:1142–1144, 1995). Because macrophages can produce both NO and superoxide anion (O_2^-), we postulated that peroxynitrite ($ONOO^-$), a product of the dilution-limited reaction of NO and O_2^- , is the candidacidal molecule of activated macrophages. We now report that $ONOO^-$, in addition to being candidacidal in vitro, is responsible for the candidacidal activity of NO-producing macrophages. $ONOO^-$ synthesis by NO-producing macrophages was triggered by two independent mechanisms: one was nonopsonic and dependent on fungal cell wall glucan moieties, and the other was dependent on opsonic antibodies. As we have demonstrated for the pathogenic fungus *C. albicans*, $ONOO^-$ may also be the molecule that enables macrophages to kill other microbes that are resistant to both O_2^- and NO.

Candida albicans is a frequent opportunistic pathogen in immunocompromised individuals. Increased incidence of *C. albicans* infections and antibiotic-resistant *C. albicans* strains underscores the need for a better understanding of the immune mechanisms that control infections by this dimorphic fungus. Nitric oxide (NO) is a well-recognized molecule of microbicidal macrophages. Recently, NO has been noted to enhance the resistance of mice to systemic as well as orogastric candidiasis and to play a role in the candidacidal activity of activated macrophages (5, 10, 30). Despite extensive experimentation, little is known about the mechanisms by which NO confers resistance to mucosal and systemic candidiasis and enhances macrophage candidacidal activity. The role of NO in macrophage candidacidal activity is apparently not a direct candidacidal effect, since macrophages from several immunodeficient mice and macrophage-like RAW 264.7 cells produced nitrite, a product of NO metabolism, but they were not candidacidal (29). In addition, NO was candidastatic but not candidacidal in vitro (29). However, the involvement of NO in macrophage candidacidal activity is clear, since inhibition of NO synthesis decreases both macrophage candidacidal activity and resistance to candidiasis (5, 10, 30).

The controversial role for NO in macrophage candidacidal activity might be explained by the chemical characteristics of this molecule. As a consequence of its unpaired electron, NO can react with multiple molecules to stimulate specific functions. A frequently studied reaction of NO is its interaction with the iron prosthetic group of guanylate cyclase, which activates the enzyme for the production of cyclic GMP (cGMP)

(27, 28). Less extensively studied is the reaction of NO and O_2^- which gives rise to the formation of peroxynitrite ($ONOO^-$), a strong oxidant able to oxidize proteins, lipids, and nucleic acids (22, 23, 25). The oxidizing properties of $ONOO^-$ can lead to diverse pathological processes, including neurotoxicity, ischemia-reperfusion injury, atherosclerosis, and sepsis (12, 13, 31, 32). Nevertheless, $ONOO^-$ may also serve as a microbicidal molecule. For example, chemically synthesized $ONOO^-$ has been shown to kill *Escherichia coli* and to inhibit *Trypanosoma cruzi* growth in vitro in a cell-free system (3, 6, 34). Macrophages can, in theory, produce large amounts of $ONOO^-$ (i.e., 500 μ M/min) (6), though only minute amounts (i.e., 0.1 nM/min) have been detected from phorbol myristate acetate (PMA)-activated macrophages (9). To date, no one has demonstrated that macrophages can use $ONOO^-$ to kill microbes.

Since *C. albicans* can enhance the respiratory burst of macrophages, it is possible that the candidacidal activity of NO-producing macrophages might be due to the production of $ONOO^-$. By using an array of oxidative molecules and several sources of macrophages in different states of differentiation, we investigated the role of $ONOO^-$ in fungicidal activity. We now demonstrate that glucan-stimulated $ONOO^-$, but not NO by itself, enhances macrophage fungicidal activity.

MATERIALS AND METHODS

Microorganisms. *C. albicans* B311 (type A), *Cryptococcus neoformans* CIA (encapsulated) and M7 (nonencapsulated), and *Saccharomyces cerevisiae* (ATCC 9763) were maintained by monthly transfer on Sabouraud dextrose agar (SDA) (Becton Dickinson, Cockeysville, Md.) slants. Before investigation, the yeast cells were cultured for 24 h at 37°C on SDA, washed from the slants, and adjusted to the desired concentration in phosphate-buffered saline (PBS) or RPMI medium (Sigma, St. Louis, Mo.) supplemented with 10% heat-inactivated fetal calf serum (FCS).

Mice. Male and female BALB/c mice between 6 and 9 weeks of age, raised under germfree conditions at the University of Wisconsin Gnotobiotic Research Laboratory, Madison, were used throughout this study.

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FIG. 1. Effect of oxidants on *C. albicans* viability. The viability of *C. albicans* was examined after its exposure to O_2^- and NO donors and $ONOO^-$ at different doses (A) and incubation times (B). The candidacidal activity of SIN-1, an $ONOO^-$ donor, was inhibited by scavengers of either NO (i.e., PTIO) or O_2^- (i.e., 1,1,3,3-tetramethyl-2-thiourea [TMTU]) (C) and was not dependent on the generation of hydroxyl radicals (D). SNP, sodium nitroprusside.

Isolation of murine macrophages. Murine resident peritoneal cells were harvested by washing the peritoneal cavity with 10 ml of sterile, ice-cold Dulbecco's PBS supplemented with 10% heat-inactivated FCS. The resident peritoneal cells were centrifuged for 15 min at $200 \times g$ at $4^\circ C$ and then resuspended in RPMI medium supplemented with 10% heat-inactivated FCS. The viability of the resident peritoneal cells was always $>95\%$ as determined by trypan blue exclusion. After the resident peritoneal cells were incubated for 4 h at $37^\circ C$, the adherent macrophages were selected by aspirating the nonadherent cells with prewarmed, FCS-supplemented RPMI medium. Over 95% of the adherent resident peritoneal cells morphologically resembled macrophages, as determined by use of Wright stain (Fisher, Pittsburgh, Pa.). Only the wells that were confluent with macrophages were used in this study. The number of effector cells was estimated microscopically from macrophages isolated from a standard well treated with EDTA and trypsin.

Activation of macrophages. Macrophages were incubated for 18 h with 200 U of recombinant murine interferon gamma ($IFN-\gamma$) (provided by Genentech, South San Francisco, Calif.) per ml and with $1 \mu g$ of lipopolysaccharide (LPS) per ml during the last 2 h of incubation. The macrophages were then incubated with yeast cells for 2 h, and their fungicidal activity was determined by quantifying surviving yeasts as described below.

Determination of fungicidal activity. The fungicidal activity of macrophages was assayed as previously described (30). Briefly, polystyrene-adherent resident peritoneal macrophages were cultured with yeast cells at an effector-to-target (E/T) ratio of 10:1 in a 96-well plate. As a control, yeast cells were cultured

without macrophages under similar conditions. To estimate the contribution of NO- and O_2^- -mediated killing, aminoguanidine (Sigma) (2), a specific inhibitor of NOS; PTIO (Sigma) (14, 33), a molecule that reacts with NO to form NO_2 ; or 5'-aminosalicylic acid (Sigma) (1), a scavenger of O_2^- , was added (0 to 1 mM) to the macrophages together with the yeast cells. Aminoguanidine, PTIO, and 5'-aminosalicylic acid were prepared in FCS-supplemented RPMI medium. After 2 h of incubation, Triton X-100 was added to the wells to give a final concentration of 0.1%. The contents of the wells were removed by vigorous pipetting and washing with sterile double-distilled H_2O . The removal of macrophages and yeast cells from the wells was confirmed with an inverted microscope. Subsequently, each sample was serially diluted and cultured on SDA plates, and the number of viable yeasts able to form a colony (CFU) was scored after overnight incubation at $37^\circ C$. The fungicidal activity was calculated as $[(CFU \text{ in control well} - CFU \text{ in test well})/CFU \text{ in control well}] \times 100$. In some experiments, the fungicidal activity of several oxidizing agents was also tested. Sodium nitroprusside (Sigma), SIN-1 (3-morpholinodisodionimine) (Calbiochem, San Diego, Calif.), and potassium dioxide (Sigma), all prepared in PBS, and chemically synthesized $ONOO^-$ (see "Peroxyntirite synthesis" below) were added to 10^4 CFU of yeast cells resuspended in PBS. The fungicidal activity of these oxidizing agents was calculated as described for the macrophages.

Nitrite determination. The production of NO by 10^5 macrophages in 4 h was estimated by measuring the accumulation of nitrite, a stable metabolite of the reaction of NO with oxygen, by the Greiss reaction. The concentration of nitrite in the supernatants from macrophages mixed with an equal volume of Greiss

FIG. 2. NO and O_2^- are involved in macrophage candidacidal activity. The candidacidal activity (□) and nitrite-producing capacity (◆) of macrophages activated with IFN- γ and LPS were studied in the presence of various concentrations of the NO synthase inhibitor aminoguanidine (A), the NO scavenger PTIO (B), and the O_2^- scavenger 5'-aminosalicylic acid (C). The candidacidal activity was estimated by enumerating the *C. albicans* organisms able to form a colony, and the nitrite was estimated by the Greiss reaction, as described in Materials and Methods.

reagent (0.5% sulfanilamide and 0.05% *N*-1-naphthylethylenediamide hydrochloride in 2.5% phosphoric acid) was estimated spectrophotometrically (550 nm) as described previously (30). The concentration of nitrite was determined from a standard curve prepared with sodium nitrite. All reagents for the nitrite determination were purchased from Sigma.

Superoxide anion determination. O_2^- was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (21). Briefly, resident peritoneal macrophages isolated as described above and macrophage-like RAW 264.7 cells grown in 10% FCS RPMI medium supplemented with L-glutamine were incubated in 60 μ M ferricytochrome *c* in phenol red-free Earle's balanced salt solution. After a 90-min incubation at 37°C in a CO_2 incubator, the optical density of the supernatants was determined spectrophotometrically at 550 nm. The concentration of O_2^- was calculated by using an E_{550} of 2.1×10^4 M⁻¹ cm⁻¹. Results are expressed as nanomolar O_2^- per milligram of protein per min. The protein was measured with a bicinchoninic acid kit as recommended by the manufacturer (Pierce, Rockford, Ill.).

Peroxyntirite determination. Macrophage ONOO⁻ production was estimated by the oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma) with a Los Alamos Diagnostics model 633 chemiluminometer as described previously (24). Resident peritoneal macrophages and RAW 264.7 cells plated on glass coverslips were stimulated with 200 U of IFN- γ for 18 h and 1 μ g of LPS per ml during the last 3 h. Subsequently, the macrophages were incubated separately with live *C. albicans*, heat-killed *C. albicans*, *S. cerevisiae*, and *C. neoformans* CIA and M7 at an E/T ratio of 1:1, 100 μ g of soluble and particulate

antigens per ml from yeast cells or hyphae of *C. albicans* (French press preparation), 1 mg of mannans (Sigma) per ml, and 1 mg of glucans (Sigma) per ml for 30 min. For some experiments, mannans or glucans were added 5 min prior to live *C. albicans*. Where indicated, *C. neoformans* was preopsonized with 10 μ g of the immunoglobulin G1 (IgG1) monoclonal antibody to glucuronoxylomannan, clone 2H1 (17, 18), per ml. The coverslips were then transferred to a cuvette containing 100 μ M luminol, and the chemiluminescence was recorded by a computer interface. The amount of ONOO⁻ produced by the macrophages was calculated from a standard curve prepared with chemically synthesized ONOO⁻.

Peroxyntirite synthesis. Peroxyntirite was synthesized as previously described (16). Briefly, an ice-cold solution of 0.6 M HCl-0.7 M H_2O_2 was added to an ice-cold solution of 0.6 M NaNO₂, and then 1.5 M NaOH was immediately added. Excess H_2O_2 was removed by addition of MnO₂. The concentration of peroxyntirite was determined by UV absorbance spectroscopy at 302 nm in 1 N NaOH ($E_{302} = 1,670$ M⁻¹ cm⁻¹). ONOO⁻ was stored at -80°C and used within 2 weeks of synthesis.

Flow cytometric analysis. The presence or absence of β -1,3-glucans on viable *C. albicans*, *S. cerevisiae*, and *C. neoformans* CIA and M7 was detected by flow cytometry with a rabbit polyclonal antiserum against β -1,3-glucan (a generous gift of K. H. Nollstadt [19]). Yeast cells, resuspended in Hanks' balanced salt solution without phenol calcium or magnesium (Biowhitaker, Walkersville, Md.) supplemented with 1% heat-inactivated fetal bovine serum, were incubated with anti β -1,3-glucan immune serum or with a nonimmune serum control at 37°C for 90 min. After a washing with the first antibody, the yeast cells were incubated with a secondary fluorescein isothiocyanate-conjugated-goat anti-rabbit IgG (heavy plus light chain) (Dynatech, Chantilly, Va.) at 37°C for 60 min. Flow cytometry was performed on a FACScan fluorescence-activated cell sorter (Becton Dickinson), and the data were collected and analyzed on a Hewlett-Packard (Palo Alto, Calif.) computer (HP 310) using LYSIS II software (Becton Dickinson).

Statistical analysis. The results are the means $\pm$ standard errors of the means of at least four independent observations from two separate experiments. Analysis of variance followed by Fisher's protected least significant difference procedure was used to determine statistical significance between control and treatment groups. A *P* of <0.05 was considered significant.

RESULTS

Effect of oxidants on *C. albicans* viability. As previously shown (29), sodium nitroprusside, an NO generator, did not kill *C. albicans* after a 16-h incubation (Fig. 1A). Conversely, O_2^- and ONOO⁻ donors (KO_2 and SIN-1, respectively) and chemically synthesized ONOO⁻ killed similar numbers of *C. albicans* organisms after a 16-h incubation (Fig. 1A). However, ONOO⁻ was a more efficient candidacidal molecule than KO_2 or SIN-1 in shorter incubations (Fig. 1B). At 1 h, 3 mM ONOO⁻ completely killed the inoculum of *C. albicans*. A higher dose (10 mM) and longer exposure time (2 h) were necessary for KO_2 and SIN-1 to sterilize a culture of 10^4 *C. albicans* organisms. The concentration of ONOO⁻ used in vitro may have biological relevance, since macrophages can, in theory, produce 500 μ M/min within the phagolysosome (6). The candidacidal activity of SIN-1 depends on the reaction of NO radicals and O_2^- (Fig. 1C). Killing of *C. albicans* by SIN-1 was inhibited by the NO scavenger PTIO and by the O_2^- scavenger 1,1,3,3-tetramethyl-2-thiourea (Sigma). Urea (Sigma), which does not scavenge O_2^- , did not inhibit the candidacidal activity of SIN-1. O_2^- and NO scavengers (*P* < 0.01), but not hydroxyl radical scavengers (*P* = 0.98), reduced the candidacidal activity of the ONOO⁻ donor SIN-1 (Fig. 1C and D). Hydroxyl radical scavengers alone did not reduce the viability of *C. albicans* (data not shown).

NO and O_2^- are involved in macrophage candidacidal activity. Aminoguanidine, an inhibitor with a high specificity for inducible NO synthase (2), reduced in a dose-dependent manner both the candidacidal activity and the nitrite-producing capacity (*P* < 0.01) of macrophages activated in vitro with IFN- γ and LPS (Fig. 2A). Nitrite production accompanied by reduced macrophage candidacidal activity occurred under two different experimental conditions. First, interference with the reaction of NO and O_2^- by PTIO inhibited macrophage candidacidal activity and significantly (*P* = 0.0001) increased the nitrite recovered from supernatants of activated macrophages

FIG. 3. Candidacidal macrophages produce ONOO⁻. (A) *C. albicans* (E/T ratio, 1:1) or PMA (20 nM) was used as a stimulant of O₂⁻ production from IFN- γ - and LPS-activated resident peritoneal macrophages and RAW 264.7 cells. The production of O₂⁻ by the macrophages was measured by the reduction of ferricytochrome and was inhibitable by superoxide dismutase. □, macrophages; ■, RAW 264.7 cells. (B) IFN- γ - and LPS-activated macrophages were also studied for the production of ONOO⁻ in response to *C. albicans* (E/T ratio, 1:1). ONOO⁻ production was measured by luminol chemiluminescence and was diminished by either the NO synthase inhibitor aminoguanidine (250 μ M) or the O₂⁻ scavenger 5'-aminosalicylic acid (1,000 μ M). □, macrophages plus *C. albicans* or macrophages plus IFN- γ and LPS; ◆, macrophages plus IFN- γ , LPS, and *C. albicans*; ○, macrophages plus IFN- γ , LPS, *C. albicans*, and aminoguanidine; △, macrophages plus IFN- γ , LPS, *C. albicans*, and aminosalicic acid; ⊞, RAW 264.7 cells plus IFN- γ , LPS, and *C. albicans*.

(Fig. 2B). Second, scavenging O₂⁻ by 5'-aminosalicylic acid decreased macrophage candidacidal activity while significantly ($P < 0.05$) increasing nitrite-producing capacity (Fig. 2C).

Candidacidal macrophages produce ONOO⁻. Stimulation of macrophages with PMA, a known stimulus for O₂⁻ production, increased their O₂⁻-producing capacity (Fig. 3A). Activated macrophages incubated with *C. albicans* produced about 80 nM O₂⁻ per mg of protein per min. This increase in O₂⁻ production could be inhibited by superoxide dismutase. *C. albicans* was required for O₂⁻ production by macrophages, since IFN- γ -primed, LPS-triggered macrophages that were not challenged with *C. albicans* did not produce any detectable O₂⁻. Activation of macrophages with IFN- γ and LPS was not required for O₂⁻ production, since unstimulated control macrophages incubated with *C. albicans* produced 55 nM O₂⁻ per mg of protein per min. In contrast to primary macrophages, RAW 264.7 cells produced low levels of O₂⁻ in response to PMA and no detectable O₂⁻ in response to *C. albicans* ($P = 0.0001$). IFN- γ -activated macrophages and *C. albicans*-challenged, unstimulated control macrophages did not produce ONOO⁻; however, addition of *C. albicans* to macrophages previously activated with IFN- γ and LPS triggered the synthesis of ONOO⁻ (Fig. 3B). Macrophage ONOO⁻ production could be reduced by the NOS inhibitor aminoguanidine and by the O₂⁻ scavenger 5'-aminosalicylic acid. RAW 264.7 cells activated with IFN- γ and LPS and then challenged with *C. albicans* produced significantly less ONOO⁻ than control resident peritoneal macrophages. Of note was the fact that activated macrophages produced more ONOO⁻ than O₂⁻. Although we don't have a good explanation for this, the fact that different methods for detection were employed and the fact that ONOO⁻ can interfere with Fe³⁺ of cytochrome *c* may have contributed to this difference.

Glucans and opsonizing antibodies trigger macrophage ONOO⁻ synthesis. Both live and heat-killed *C. albicans* triggered macrophage ONOO⁻ synthesis (Fig. 4A). In contrast to soluble cytoplasmic fractions, cell wall-enriched particulate fractions from either *C. albicans* blastoconidia or hyphae stimulated ONOO⁻ synthesis by macrophages activated previously with IFN- γ and LPS. Cell wall glucans triggered macrophage

ONOO⁻ production (Fig. 4B). Glucans stimulated 10 μ M ONOO⁻ per mg of macrophage protein per min (significant increase [$P < 0.0001$] versus control macrophages). Moreover, preincubation of activated macrophages with glucans significantly ($P < 0.005$) decreased their ONOO⁻-producing capacity in response to *C. albicans*. In contrast to glucans, mannans, another important cell wall component of fungal cells, did not modulate ($P = 0.8$) the capacity of activated macrophages to produce ONOO⁻.

To further test the role of glucans in the activation of macrophage ONOO⁻ production, we used several fungal species with different β -1,3-glucan surface expression (Table 1). *C. albicans*, *S. cerevisiae*, and nonencapsulated *C. neoformans* M7 expressed surface β -1,3-glucan, were phagocytized, stimulated ONOO⁻ production, and were killed by activated macrophages. Opsonization of encapsulated *C. neoformans* CIA, which did not express surface β -1,3-glucans, with an IgG1 monoclonal antibody specific for glucuronoxylomannan enhanced phagocytosis, ONOO⁻ production, and fungicidal activity of IFN- γ -activated macrophages. *S. cerevisiae* and IgG1-opsonized, encapsulated *C. neoformans* stimulated more O₂⁻ production from unstimulated macrophages than either *C. albicans* or nonencapsulated or encapsulated *C. neoformans*. All of the fungi tested were susceptible to chemically synthesized ONOO⁻-mediated killing.

DISCUSSION

The mechanisms of action of NO can be broadly classified as cGMP dependent or cGMP independent (28). We observed here that the increased macrophage candidacidal activity of NO-producing macrophages is not likely to be a cGMP-dependent process, since cGMP was not increased when measured by radioimmunoassay (Amersham kit; data not shown). Instead, killing of *C. albicans* by NO-producing macrophages is a cGMP-independent mechanism that relies on the formation of ONOO⁻ from NO and O₂⁻. The observation that *C. albicans* was killed by the ONOO⁻ generator SIN-1 and by chemically synthesized ONOO⁻ demonstrates that ONOO⁻ is candidacidal. Killing of *C. albicans* by SIN-1 was dependent on the

FIG. 4. Glucans in the cell wall of *C. albicans* trigger macrophage ONOO⁻ production. IFN- γ - and LPS-activated macrophages were incubated alone (control) (□) or with live *C. albicans* (E/T ratio, 1:1) (◇), heat-killed *C. albicans* (E/T ratio, 1:1) (○), or 100 μ g of soluble (◻) and particulate (△) antigens from yeast cells or hyphae of *C. albicans* per ml (A) or with mannans (1 mg/ml) and glucans (1 mg/ml) (B) for 30 min. ONOO⁻ production was measured by luminol.

generation of both NO and O₂⁻ and was a direct consequence of the strong oxidizing nature of ONOO⁻ and not its ability to generate hydroxyl radicals. In addition to killing *C. albicans* in vitro, ONOO⁻ also appears to be responsible for the candidacidal activity of NO-producing macrophages. The fact that inhibitors of NO synthesis and scavengers of either NO or O₂⁻ reduced macrophage candidacidal activity supports the latter hypothesis.

We also explored the role of ONOO⁻ in macrophage candidacidal activity by comparing its production by macrophages with similar NO-producing characteristics but different candidacidal activities (29). RAW 264.7 cells, which have low candidacidal activity, produced less O₂⁻ and ONOO⁻ when challenged with *C. albicans* than highly candidacidal resident peritoneal macrophages. The small amounts of ONOO⁻ produced by RAW 264.7 cells may explain their poor candidacidal activity (29) and are consistent with the hypothesis that ONOO⁻ is an effector molecule of NO-producing macrophages. Evidence from two separate experiments indicated that killing of *C. albicans* required an adequate amount of ONOO⁻. First, 3 mM chemically synthesized ONOO⁻, but not 1 mM, was candidacidal in vitro, and second, candidacidal macrophages produced approximately 110 μ M ONOO⁻ per min, whereas noncandidacidal macrophages (i.e., RAW 264.7 cells) produced about 34 μ M/min.

Different susceptibilities of pathogenic yeasts to oxidants might be a determinant of microbial virulence. For instance, *S. cerevisiae*, a nonpathogenic fungus, was efficiently killed by unstimulated O₂⁻-producing macrophages, whereas *C. albi-*

cans was not. Although O₂⁻ has been associated with candidacidal activity (present study and reference 26), it is not likely that O₂⁻ is a killing molecule by itself at the concentrations at which macrophages produce it (present study and reference 26). Killing of pathogenic *C. albicans* appears to require the generation of strong oxidant molecules. The susceptibility of *C. albicans* to O₂⁻-derived metabolites of the myeloperoxidase system of neutrophils and monocytes is well characterized (11, 15). Because myeloperoxidase is lost during the differentiation of monocytes into macrophages (15), there must exist other pathways that generate strong oxidants from O₂⁻. Analogous to the chloramines produced by the myeloperoxidase system of neutrophils and monocytes, synthesis of ONOO⁻ by the chemical reaction of O₂⁻ and NO may enable macrophages to generate a strong oxidant able to kill pathogenic microorganisms that are resistant to O₂⁻ and NO.

Macrophages can generate an extensive array of oxidative molecules with potential cytotoxicity for a variety of microbes. Of these oxidative molecules, NO has been shown to potentiate the in vitro killing of *E. coli* (in a cell-free system) by hydrogen peroxide (20). Hydrogen peroxide is a product of the dismutation of O₂⁻ by superoxide dismutase. Because O₂⁻ reacts three times faster with NO than with superoxide dismutase (3, 23), in the presence of NO the majority of O₂⁻ forms ONOO⁻ (9). Thus, we conclude that the candidacidal activity of IFN- γ -activated macrophages is mainly the result of the candidacidal characteristics of ONOO⁻.

Production of ONOO⁻ required the interaction of activated macrophages and fungal cell walls. One of the fungal cell wall

TABLE 1. Stimulation of macrophage ONOO⁻ synthesis by fungi with different β -1,3-glucan-expressing characteristics

Organism	Expression of β -1,3-glucan	Phagocytosis	ONOO ⁻ (μ M/mg/min)	O ₂ ⁻ (nM/mg/min)	Fungicidal activity (%)		
					Macrophages		ONOO ⁻ (3 mM)
					IFN + LPS	Control	
<i>C. albicans</i>	+	+	112	55	71	12	92
<i>S. cerevisiae</i>	+	+	64	76	98	86	100
M7 ^a	+	+	82	17	69	37	100
CIA ^b	-	-	1	4	3	0	100
CIA + IgG1	ND ^c	+	22	124	68	50	ND

^a Nonencapsulated *C. neoformans* strain.

^b Encapsulated *C. neoformans* strain.

^c ND, not determined.

moieties that triggered macrophage ONOO⁻ production belongs to the structural carbohydrate glucan. Glucan by itself stimulated small, but significant ($P < 0.001$), amounts of ONOO⁻. Furthermore, this moiety substantially reduced the ability of *C. albicans* to trigger macrophage ONOO⁻ production, which suggests that cross-linkage of glucan receptors is necessary for the production of ONOO⁻. Glucans can bind to the complement receptor CR3 and stimulate O₂⁻ synthesis (8), which in turn may react with the continuously formed NO to generate the fungicidal ONOO⁻ molecule. Alternatively, glucans, by promoting the binding of yeasts to macrophages (7), may facilitate the coupling of other yeast ligands with other macrophage receptors for the generation of ONOO⁻.

To further test the role of glucans in the stimulation of macrophage ONOO⁻ synthesis, we used fungi with different levels of β -1,3-glucan surface expression. β -1,3-Glucan-expressing *C. albicans*, *S. cerevisiae*, and nonencapsulated *C. neoformans* M7 were phagocytized, triggered ONOO⁻ and O₂⁻ synthesis, and were killed by activated macrophages. In contrast, virulent encapsulated *C. neoformans* CIA, which has a capsule rich in glucuronoxylomannan that blocks β -1,3-glucan surface expression, was not phagocytized, did not trigger ONOO⁻ and O₂⁻ synthesis, and was not killed by macrophages. These studies with β -1,3-glucan-expressing fungi support the hypothesis that fungal cell glucan moieties trigger macrophage synthesis of O₂⁻, which in turn reacts with the continuously formed NO to produce ONOO⁻. Together with glucans, other fungal cell wall moieties very likely contributed to the stimulation of macrophage ONOO⁻ synthesis as well.

Furthermore, we show that in the absence of exposed glucans (i.e., as is the case for encapsulated *C. neoformans*), Fc receptor coupling also contributes to the release of ONOO⁻ by activated macrophages. Stimulation of macrophage ONOO⁻ and O₂⁻ synthesis by opsonized *C. neoformans* might explain why antibodies to glucuronoxylomannan enhanced phagocytosis and killing of *C. neoformans* by macrophages and increased the resistance of mice to this opportunistic fungus (17, 18). Our studies with encapsulated and nonencapsulated *C. neoformans* demonstrate that macrophage ONOO⁻ production can be stimulated by at least two separate mechanisms. Nevertheless, the concentration of ONOO⁻ synthesized by activated macrophages appears to depend on the route by which a microbe is interiorized; glucans triggered macrophage ONOO⁻ production more efficiently than opsonizing antibodies. At the present time, it remains unclear why antibody-mediated phagocytosis triggered low levels of macrophage ONOO⁻ production, since the levels of NO and O₂⁻ synthesis were high.

Production of NO in association with a function does not identify NO as the effector molecule of that function. Thus, some of the toxic mechanisms that were ascribed to NO, such as inactivation of aconitase, are now attributed to ONOO⁻ (4). In addition to this molecular selectivity, our studies with *C. albicans* identify a microorganism that is specifically killed by ONOO⁻ but not by NO. Moreover, not only do our studies demonstrate that ONOO⁻ can mediate macrophage candidacidal activity, but they have also identified glucans associated with the fungal cell wall and opsonizing antibodies as important determinants for the stimulation of ONOO⁻ in NO-producing macrophages. Because a variety of microbes can elicit O₂⁻ synthesis in NO-producing macrophages, it is possible that ONOO⁻ is a common component of the antimicrobial armamentarium of activated macrophages.

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