Oxidative Responses of Human and Murine Macrophages During Phagocytosis of Leishmania chagasi

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Leishmania chagasi, the cause of South American visceral leishmaniasis, must survive antimicrobial responses of host macrophages to establish infection. Macrophage oxidative responses have been shown to diminish in the presence of intracellular leishmaniasis. However, using electron spin resonance we demonstrated that murine and human macrophages produce \( \text{O}_2^- \) during phagocytosis of opsonized promastigotes. Addition of the \( \text{O}_2^- \) scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl to cultures resulted in increased infection, suggesting that \( \text{O}_2^- \) enhances macrophage leishmanicidal activity. The importance of \( \text{NO} \) produced by inducible NO synthase (iNOS) in controlling murine leishmaniasis is established, but its role in human macrophages has been debated. We detected \( \text{NO}^- \) in supernatants from murine, but not human, macrophages infected with \( L. \) chagasi. Nonetheless, the iNOS inhibitor N\(^\omega\)-monomethyl-L-arginine inhibited IFN-\( \gamma \)-mediated intracellular killing by both murine and human macrophages. According to RNase protection assay and immunohistochemistry, iNOS mRNA and protein were expressed at higher levels in bone marrow of patients with visceral leishmaniasis than in controls. The iNOS protein also increased upon infection of human macrophages with \( L. \) chagasi promastigotes in vitro in the presence of IFN-\( \gamma \). These data suggest that \( \text{O}_2^- \) and \( \text{NO}^- \) each contribute to intracellular killing of \( L. \) chagasi in human and murine macrophages. The Journal of Immunology, 2001, 167: 893–901.

Leishmania sp. are obligate intracellular protozoa that infect and replicate within mammalian macrophages. This parasite is dimorphic, having an extracellular promastigote form in the sandfly vector that converts into an intracellular amastigote within the macrophage. Flagellated promastigotes are delivered into a blood pool at the site of the sandfly bite. Promastigotes are ingested by mammalian mononuclear phagocytes, whereupon they lose their flagella and transform into amastigotes. Amastigotes survive and replicate within phagolysosomes. Intracellular amastigotes can eventually cause clinical manifestations of leishmaniasis, which range from cutaneous ulcers to a potentially fatal visceral disease. Visceral leishmaniasis is generally caused by \( L. \) chagasi, \( L. \) donovani, or \( L. \) infantum (1).

The macrophage is armed with antimicrobial mechanisms that intracellular organisms must evade to survive. During leishmaniasis the microbicidal interactions between parasite and host cells occur in two stages. First, during initial phagocytosis of promastigotes the macrophage can undergo an oxidative response stimulated by the phagocytosis event. Second, once infection with amastigotes is established, the quiescent macrophage can be activated to potentially kill intracellular leishmania. Efficient evasion of toxic microbicidal molecules produced at each stage of infection is important for leishmania to be able to initiate and maintain host cell infection.

Two important macrophage-derived oxidants have been identified as critical in controlling leishmaniasis infection. During the first stages of infection superoxide (\( \text{O}_2^- \)) is produced as part of the respiratory burst of human and murine macrophages in response to phagocytosis (2, 3). \( \text{O}_2^- \) production is catalyzed by the NADPH oxidase, a heme-containing cytochrome that contains cytosolic and membrane-bound components. Once assembled the oxidase transfers an electron from NADPH to molecular oxygen, producing \( \text{O}_2^- \). \( L. \) chagasi promastigotes have been shown to be susceptible to killing by exposure to \( \text{O}_2^- \) and hydroxyl radical (\( \cdot \text{OH} \)) generated from \( \text{H}_2\text{O}_2 \) (4, 5).

A second anti-leishmanial oxidant produced by macrophages is \( \text{NO}^- \) (6–8). Unlike \( \text{O}_2^- \), which is a generated during phagocytosis of the parasite, \( \text{NO}^- \) is generated after macrophage activation by IFN-\( \gamma \) and TNF-\( \alpha \) and is most relevant to killing established intracellular amastigotes. Inhibitors such as N\(^\omega\)-monomethyl-L-arginine (\( L\)-NMMA)\(^3\) lead to an increase in amastigote survival and replication in murine macrophages (9). Although there is strong evidence that \( \text{NO}^- \) plays an important role in murine leishmaniasis, it remains controversial whether \( \text{NO}^- \) plays a role in the anti-leishmanial responses of human macrophages (10, 11). NO\(^-\) was reported to participate in the killing of \( L. \) major by human macrophages that are stimulated through the low affinity Fc\( \varepsilon \) receptor, CD23, and IFN-\( \gamma \) (11). Furthermore, inducible NO synthase (iNOS) is produced in alveolar macrophages from patients infected...
with *Mycobacterium tuberculosis*, another intracellular micro-organism (12). Thus, there is emerging evidence suggesting that NO is generated and could participate in killing intracellular microbes by human macrophages.

Macrophages containing intracellular *L. donovani* amastigotes have impaired antimicrobial responses, signaling, and cell surface marker expression (13, 14). This could be due in part to impaired Ca^{2+} mobilization and impaired phosphorylation of protein kinase C, events that are essential for NADPH oxidase activation and phagocytosis (15, 16). Intracellular amastigotes also cause impaired IFN-γ signaling, as does the lipophosphoglycan (LPG) isolated from promastigote membranes. It is unclear whether oxidant antimicrobial responses are inhibited by promastigotes during phagocytosis (17–19).

Recognizing that there are differences between leishmanial infection of murine vs human macrophages, the work presented in this manuscript contrasts human and murine macrophage responses to *L. chagasi*, the cause of South American visceral leishmaniasis. Using sensitive techniques for detecting O₃ and NO, we documented differences in the amount of oxidants generated after phagocytosis of *L. chagasi* promastigotes. Despite these differences, both oxidants were found to have functional consequences during leishmanial infection of murine and human macrophages.

**Materials and Methods**

**Isolation of human and murine macrophages**

Human mononuclear cells were isolated from whole blood of normal healthy donors by density sedimentation (Histopaque-1077; Sigma, St. Louis, MO). Monocytes were separated by adherence. After 5 days of culture in RPMI 1640 with 10% heat-inactivated FCS, 100 U penicillin/ml, and 100 U streptomycin/ml (RPMI-10; University of Iowa Hybridoma facility) at 37°C in 5% CO₂, adherent cells assumed characteristics of monocye-derived macrophages (MDMs). Bone marrow was removed from C3H.HJeJ (The Jackson Laboratory, Bar Harbor, ME) mouse long bones and cultured for 5–7 days in RPMI-10 supplemented with 20% L929 cell supernatant (American Type Culture Collection, Manassas, VA). Bone marrow-derived macrophages (BMMs) were harvested with 0.051 trypsin and 0.1% EDTA, and 1 × 10⁶ cells were allowed to adhere to 24-well plates or on glass coverslips overnight before use.

**Parasite culture**

A strain of *L. chagasi* (MHOM/BR/00/1669), originally isolated from a Brazilian patient with visceral leishmaniasis, was maintained by serial passage through hamsters as previously described (20). Amastigotes were recovered from hamster spleens and allowed to convert to promastigotes in hamster MEM containing 10% heat-inactivated-FCS and 80°C for up to 1 wk. DMPO spin adducts remain stable under these conditions. Electron spin resonance (ESR) spectra were obtained at room temperature using a Bruker ESP 300 spectrometer (Karlsruhe, Germany). Instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.892 G; sweep time, 0.238 G/Min; and response time, 0.655 s. Spectra detected using DMPO-OH spin adduct (ΔM = ΔH = 14.9 G). Superoxide dismutase (SOD; Sigma) or catalase was added to some macrophage cultures to differentiate between DMPO-OH generated from O₂⁻ and hydroxyl radical, respectively.

**NO detection**

BMMs and MDMs (1 × 10⁶) were cultured in 96-well round-bottom tissue culture plates (Costar, Corning, NY), and NO was quantified by the concentration of its product nitrite (NO₂⁻) in supernatants, using a NOA 280 NO analyzer (Sievers, Boulder, CO). Nitrite was reduced to NO in the presence of 1% KI and glacial CH₃OH before its detection. Some NOA wells were then treated with catalase or superoxide dismutase (SOD; Sigma) before its detection. Unless otherwise indicated, opsonized parasites were added to macrophages at a ratio of five parasites to one macrophage. Some wells contained 100 U murine IFN-γ/ml or 400 U human IFN-γ/ml (R&D Systems, Minneapolis, MN). One nanogram of LPS/ml (Sigma) and 10 μg IL-1/ml (R&D Systems) without promastigotes were added to generate positive control supernatants. Supernatants collected from murine BMMs and human MDMs were read 72 h after infection, and the concentration of NO was determined.

**Infection assays**

Human MDMs or murine BMMs (5 × 10⁵) in 10% heat-inactivated FCS/ RPMI 1640 (RP-10) were cultured on round glass coverslips (Fisher Scientific, Pittsburgh, PA) in each well of a 24-well plate (Costar, Corning, NY). Opsonized or unopsonized promastigotes at different ratios were added for 2 h and removed by washing. All conditions were performed in duplicate. Slides were collected at different time points and stained with Diff-Quik (Dade-Behring, Newark, DE). The ratio of attached or intracellular parasites to macrophages was quantified microscopically, counting a minimum of 200 cells/cover slip.

The microbicidal effects of O₂⁻ or NO were inhibited with the O₂ scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) or the NO scavenger 1-NNMA, respectively (21). To inhibit NO production, L-NMMA, 1 mM, was added to macrophages at a ratio of five parasites to one macrophage. Some wells contained 100 U human IFN-γ/ml, 0.894 G; sweep time, 0.238 G/min; and response time, 0.655 s. Spectra were obtained at room temperature using a Bruker ESP 300 spectrometer (Karlsruhe, Germany). Instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.892 G; sweep time, 0.238 G/Min; and response time, 0.655 s. Spectra detected using DMPO-OH spin adduct (ΔM = ΔH = 14.9 G). Superoxide dismutase (SOD; Sigma) or catalase was added to some macrophage cultures to differentiate between DMPO-OH generated from O₂⁻ and hydroxyl radical, respectively.

**Spin trapping**

MDMs or BMMs (1 × 10⁶) were incubated for infection for 30 min with opsonized or unopsonized promastigotes at different parasite:macrophage ratios in HBSS containing 100 μM diethylenetriaminepentacetic acid (DTPA; Sigma) to chelate iron and 100 mM of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Sigma) at 37°C in 5% CO₂. Supernatants were collected, snap-frozen, and stored at −80°C for up to 1 wk. DMPO spin adducts remain stable under these conditions. Electron spin resonance (ESR) spectra were obtained at room temperature using a Bruker ESP 300 spectrometer (Karlsruhe, Germany). Instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.892 G; sweep time, 0.238 G/Min; and response time, 0.655 s. Spectra detected using DMPO-OH spin adduct (ΔM = ΔH = 14.9 G). Superoxide dismutase (SOD; Sigma) or catalase was added to some macrophage cultures to differentiate between DMPO-OH generated from O₂⁻ and hydroxyl radical, respectively.

**RNase protection assay**

RNA was extracted from human bone marrow aspirates using RNA-STAT (Tel-Test, Friendswood, TX) following the protocol provided by the manufacturer. Human bone marrow aspirates were obtained from Brazilians hospitalized with active visceral leishmaniasis prior to or within 48 h after initiating therapy. Normal Iowan bone marrow donors served as a source of control samples (provided by Roger Gingrich, University of Iowa, Ames, IA). The custom template, in vitro transcription probes, and RNase protection kit were obtained from BD PharMingen (San Diego, CA). Two micrograms of RNA were hybridized with 1[³²P]UTP-labeled probes, RNase digested, and separated on a 5% polyacrylamide-urea gel using procedures recommended by the manufacturer. The intensity of the bands was quantified with a phosphorimager (Packard-Canberra, Meriden, CT) and calculated from cpm.

**Immunohistochemistry**

Bone marrow smears from Brazilian patients or normal donors were air-dried and fixed with 2% paraformaldehyde in PBS (20 min, 4°C). Human MDMs were infected with opsonized *L. chagasi* promastigotes at a 5:1 ratio and similarly fixed. Samples were blocked in 2% heat-inactivated FCS in Earle’s balanced salt solution, pH 7.4 (Sigma). After permeabilizing with 0.1% saponin buffer in Earle’s balanced salt solution, slides were incubated for 30 min with rabbit polyclonal IgG recognizing human iNOS (4 μg/ml saponin buffer; Santa Cruz Biotechnology, Santa Cruz, CA) in a humidified chamber. Inducible NOS was detected by incubating in alkaline phosphatase-labeled anti-rabbit IgG followed by alkaline phosphatase substrate plus levamisole according to the manufacturer’s protocol (Vector Laboratories, Burlingame, CA). Slides were counterstained with Nuclear Fast Red (Vector Laboratories), dehydrated, and visualized with light microscopy.
**MTT assay**

Promastigotes (2 x 10^5) in 100 μl HBSS were exposed to increasing doses of menadione (Sigma) or Sulfo-NONOate (Alexis, San Diego, CA) in 96-well plates at 26°C. All conditions were performed in triplicate. After 1 h 10% heat-inactivated FCS was added. Parasite viability was measured by incubation in 0.5 mg/ml MTT (Sigma) for 3 h, followed by addition of 100 μl 0.04 N HCl in isopropanol. Living mitochondria convert MTT to dark blue formazan that is soluble in acid-isopropanol and detectable on a microplate reader at 570 nm. The percentage of viability was calculated from OD readings in wells with menadione or Sulfo-NONOate compared with those in wells without these.

**2’,7’-Dichlorodihydrofluorescein diacetate (DCF) assay**

The fluorescent compound DCF was used to measure the production of reactive oxygen intermediates (ROI), including O_2·−, during promastigote phagocytosis. Five x 10^5 human MDMs or murine BMMs were suspended in HBSS containing 25 μM DCF (Molecular Probes, Eugene, OR). After 45 min at room temperature, a stimulus was added. This consisted of unopsonized or opsonized L. chagasi promastigotes at a 5:1 promastigotes-macrophages ratio, 40 μg opsonized zymosan/ml, or buffer. Macrophages were added to a 96-well microtiter plate at 4°C, and the volume was adjusted to 200 μl with 25 μM DCF in HBSS. Fluorescence due to the generation of reactive oxygen species was detected at 485 nm excitation and 538 nm emission. Emissions were monitored every 30 s at 37°C for 50 min on a BMG FLUOstar 403 microplate spectrofluorometer (BMG Lab Technologies, Durham, NC) in the Cell Fluorescence Core Facility at the Iowa City Veterans Affairs Medical Center.

**Measurement of O_2·− by ferricytochrome c reduction**

O_2·− generated after addition of opsonized zymosan to macrophages was measured in the absence or the presence of TEMPO. Macrophages (5 x 10^5) were incubated with 60 μM ferricytochrome c (Sigma). After addition of stimuli the A_550 was monitored continuously (23). The reference cuvette contained the same reagents with 62.5 μg/ml SOD.

**Results**

**Toxicity of O_2·− and NO· for L. chagasi promastigotes**

*L. chagasi* promastigotes were incubated with increasing concentrations of menadione, a redox-cycling compound that generates O_2·− and H_2O_2 (24, 25). Other promastigotes were incubated with Sulfo-NONOate, which generates NO· and has a half-life of 24 min at 25°C (26). Both compounds had a dose-dependent toxic effect on promastigotes (Fig. 1), indicating that *L. chagasi* promastigotes are susceptible to killing by O_2·−/H_2O_2 and NO·.

**EPR spectra of O_2·− production by human and murine macrophages**

To determine whether human and murine macrophages each produce O_2·− in response to *L. chagasi* promastigote phagocytosis, BMMs or MDMs were allowed to phagocytose serum-opsonized or unopsonized promastigotes in the presence of the spin trap DMPO. O_2·− produced during phagocytosis reacts with DMPO to generate DMPO-OH, which spontaneously decays to DMPO-OH and is detectable by ESR. Negative controls included medium with DMPO alone, macrophages alone plus DMPO, or parasites alone plus DMPO. Positive control macrophages were incubated with opsonized zymosan, a potent stimulus of the respiratory burst. DTPA was included with all conditions to chelate iron contaminants that might catalyze hydroxyl radical formation (27).

**Opsonization presumably alters the mechanism of receptor-mediated entry through which promastigotes undergo phagocytosis** (22, 28, 29). We questioned whether the difference between O_2·− generated during phagocytosis of opsonized or unopsonized promastigotes simply reflected a fewer number of parasites taken up. Intracellular parasites were quantified microscopically 30 min after addition of different promastigote-BMM or MDM ratios. These data are plotted on the x-axis of Fig. 3 as parasites phagocytosed per 100 macrophages. Data for the highest parasite-macrophage ratio tested for each condition are marked. Macrophages consistently took up more opsonized than untreated organisms at the same parasite-macrophage ratio, and this difference was greatest in MDMs. Plotted on the y-axis are the heights of the second low field DMPO-OH peaks on ESR spectra, normalized so that the arbitrary

![FIGURE 1. Toxic effects of O_2·− and NO· on L. chagasi promastigotes. L. chagasi promastigotes (2 x 10^5) were exposed for 1 h to increasing concentrations of either menadione or Sulfo-NONOate as indicated. Viability was determined spectrophotometrically by the conversion of MTT to formazan. Values were calculated as the percentage of MTT uptake by control parasites incubated without the above compounds. The mean ± SE percent viability values are representative of four assays for menadione or three assays for Sulfo-NONOate, each performed with triplicate conditions.](http://www.jimmunol.org/)

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Murine Bone Marrow Macrophages

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FIGURE 2. ESR spectra of O$_2^-$ produced by 1 x 10$^6$ macrophages during phagocytosis of L. chagasi promastigotes. Murine BMMs (left panel) or human MDMs (right panel) were infected for 30 min with serum-opsonized (+OPM) or unopsonized (+PM) promastigotes at a ratio of five parasites to one macrophage in the presence of the spin trap DMPO. ESR spectra of supernatants reflecting detection of the DMPO-OH spin adduct are shown. Negative controls included DMPO alone, BMM, MDM, and OPM alone. A positive control included serum-opsonized zymosan with BMMs (+OZ, left panel). +OPM+SOD (right panel) shows spectra from MDMs incubated with opsonized promastigotes in the presence of SOD, and +OPM+Cat includes MDMs incubated with opsonized promastigotes in the presence of catalase. The scale shown is Gauss units. All conditions contain DMPO and DTPA (to chelate free iron). Spectra shown are representative of at least three experiments.

unit scales are the same in the upper and lower panels. Two observations can be made from the figure. First, spectral peaks generated by MDMs were consistently higher than peaks generated by BMMs, reflecting a greater amount of O$_2^-$ generated per cell by MDMs. Second, the amount of O$_2^-$ did not continue to rise with increasing numbers of parasites phagocytosed, but instead peaked at a 5:1 or 10:1 ratio. These data suggest that differences in the amount of O$_2^-$ generated after phagocytosis of opsonized vs unopsonized parasites is not merely due to differences in the number of parasites internalized.

The DCF assay of ROI led us to the same conclusions as the ESR data. DCF provides a continuous method for measuring the amount of O$_2^-$ and other oxygen-derived radicals produced over time after addition of promastigotes to macrophages. The difference in fluorescence at sequential time points is proportional to the amount of O$_2^-$ generated during that time. As O$_2^-$ is generated spontaneously by respiring cells, fluorescent units generated by macrophages without particle addition were subtracted from fluorescence generated by cells after addition of promastigotes. The mean fluorescence generated between 30–40 min after addition of 5:1 opsonized promastigotes to MDMs was 92.4 ± 13.9 fluorescence units, compared with 17.62 ± 16.3 U generated after addition of 5:1 unopsonized promastigotes. The mean slope after addition of 5:1 opsonized promastigotes to BMMs was 11.2 ± 12.4 fluorescence units compared with 1.87 ± 7.30 fluorescence units after addition of 5:1 unopsonized promastigotes to BMMs. Thus, ROI generated during promastigote phagocytosis by MDMs was significantly greater than ROI generated by BMMs ($p < 0.001$). ROI generated by MDMs during phagocytosis of opsonized promastigotes was significantly greater than ROI generated during phagocytosis of unopsonized parasites ($p < 0.001$). Differences for BMMs did not achieve statistical significance, presumably because of the low absolute values.

**NO$^\cdot$ detection in human and murine samples**

To determine whether detectable NO$^\cdot$ is produced during infection of murine or human macrophages with L. chagasi, we measured NO$^\cdot$ produced from NO$^\cdot$ released into culture supernatants of infected cultures. IFN-γ is necessary for leishmanicidal activity and transcription of iNOS. Therefore, 100 U recombinant murine IFN-γ/ml or 400 U recombinant human IFN-γ/ml were added before measuring NO$^\cdot$ (9). To maximally stimulate NO$^\cdot$ production by murine BMMs, IFN-γ, IL-1, and LPS were added to some conditions.

BMMs infected with L. chagasi promastigotes plus IFN-γ for 72 h showed increased NO$^\cdot$ production over the IFN-γ treatment alone (Fig. 4). NO$^\cdot$ was also detected at 48 h (Fig. 4), but not at 4 h (data not shown), after infection. Consistent with some prior studies, nitrite resulting from NO$^\cdot$ generation was not detected in human MDMs infected with L. chagasi (10) even in the presence of stimulatory cytokines. Furthermore, after fully reducing supernatants to detect any NO$^\cdot$, NO$^\cdot$ was still not detected in human cells stimulated with IFN-γ, LPS, or in the presence of IFN-γ and parasites. The amounts of NO$^\cdot$ generated by BMMs infected with opsonized vs unopsonized promastigotes were similar.

**Macrophage infection in the presence of O$_2^-$ and NO$^\cdot$ inhibitors**

To document the importance of O$_2^-$ and NO$^\cdot$ in leishmanicidal killing by human and murine macrophages in vitro, infection assays were conducted in the presence of inhibitors of both oxidant species.
MDM data are represented in the same units. Peak heights are shown in arbitrary units, normalized so that BMM and MDMs were infected with different ratios of serum-opsonized or unopsonized promastigotes to macrophages (1:1, 5:1, 10:1, and 20:1). The highest ratio tested is indicated next to the points with the highest numbers of intracellular parasites. Thirty minutes later the number of parasites per 100 macrophages was quantified after 24 h, so that viable and dead parasites could be distinguished microscopically. Incubation in 0.4 mM TEM-FIGURE 3. $O_2^-$ produced during macrophage infection with different numbers of opsonized vs unopsonized promastigotes. Murine BMMs (1 × 10^6; top panel) or human MDMs (bottom panel) were infected with different ratios of serum-opsonized or unopsonized promastigotes to macrophages (1:1, 5:1, 10:1, and 20:1). The highest ratio tested is indicated next to the points with the highest numbers of intracellular parasites. Thirty minutes later the number of parasites per 100 macrophages was quantified microscopically, as shown on the x-axis. These data are plotted against the magnitude of the second low field DMPO-OH peaks on ESR spectra generated during the first 30 min after phagocytosis of promastigotes. The ESR peak heights are shown in arbitrary units, normalized so that BMM and MDM data are represented in the same units.

Human MDMs and murine BMMs were infected with opsonized promastigotes in the presence of TEMPOL, an $O_2^-$ scavenger that crosses cell membranes and therefore can be used to scavenge $O_2^-$ in living phagocytes (21). Although the respiratory burst occurs within 30 min of phagocytosis (30), intracellular parasites were quantified after 24 h, so that viable and dead parasites could be clearly distinguished microscopically. Incubation in 0.4 mM TEMPOL during phagocytosis significantly increased the level of intracellular infection of human MDMs ($p < 0.05$; Fig. 5A). TEMPOL did not appear to enhance phagocytosis, because the number of parasites ingested in the presence of TEMPOL was approximately equal 1 h after infection (data not shown). Similarly, murine cells showed increased infection when incubated with TEMPOL, although the differences did not reach statistical significance ($p = 0.08$; $n = 4$ experiments; Fig. 5A). This could be due to the fact that murine macrophages produce less $O_2^-$ than human cells upon phagocytosis of opsonized parasites (Figs. 2 and 3).

3). IFN-γ significantly augmented parasite killing by murine and human cells 48 h after its addition (Fig. 5B). The difference was not apparent at 24 h (Fig. 5A), probably because an IFN-γ-mediated increase in microbial activity was not evident at this early time point.

The ability of TEMPOL to scavenge $O_2^-$ generated during phagocytosis of opsonized zymosan by human MDMs was confirmed using the ferricytochrome c reduction assay to detect $O_2^-$ (23). The change in $A_{550}$ over 15 min after addition of opsonized zymosan to MDMs was 0.120 in the absence vs 0.006 in the presence of 0.4 mM TEMPOL. A 1/1000 dilution of ethanol, the concentration of the TEMPOL solvent, did not induce BMM toxicity, as measured by exclusion of trypan blue. Furthermore, TEMPOL itself was not toxic for MDMs or BMMs at concentrations of 1 mM or lower.

The iNOS inhibitor L-NMMA has been shown to increase leishmanial survival in murine macrophages, but it has been reported that L-NMMA does not affect parasite survival in murine macrophages (10, 31, 32). To test the importance of NO for intracellular killing of L. chagasi in murine macrophages, L-NMMA was quantified after infection in the presence of IFN-γ with or without L-NMMA. Infected macrophages with L-NMMA resulted in a significantly higher parasite burden compared with untreated control BMMs at 48 h after infection (Fig. 5B). When MDMs were preinfected with L. chagasi for 48 h to allow their conversion to amastigote forms, treatment with L-NMMA for a subsequent 48 h significantly inhibited intracellular killing of parasites by human macrophages. L-NMMA inhibited NO detected in BMM cultures stimulated with IFN-γ and LPS by 74.8 ± 2.7%, indicating that the inhibitor was active. These data suggest that although NO production by human cells after L. chagasi infection is not detectable according to the current methods available, NO is nonetheless helping to control L. chagasi infection.
Expression of iNOS mRNA in bone marrow aspirates from Brazilian patients with visceral leishmaniasis

To determine whether humans could produce NO' during L. chagasi infection, we studied the expression of iNOS in mRNA extracted from bone marrow aspirates of Brazilian patients with visceral leishmaniasis using an RNase protection assay (Fig. 6). The two visceral leishmaniasis patients represented had their aspirates before (lane 1) or after 1 day (lane 2) of therapy. Aspirates from healthy Iowan bone marrow transplant donors served as controls (lanes 3 and 4). The ratio of iNOS to GAPDH mRNA was 0.245 ± 0.007 (mean ± SD) in visceral leishmaniasis patients vs 0.125 ± 0.035 in control samples.

Expression of iNOS protein in human MDMs in vitro and in bone marrow from Brazilian patients with visceral leishmaniasis

Immunohistochemistry was used to document the presence of iNOS protein in bone marrow smears from visceral leishmaniasis patients and in infected MDMs (Fig. 7). Inducible NOS was found in nucleated cells in the bone marrow of patients with visceral leishmaniasis (see a representative marrow smear in Fig. 7B; n = 4). This appeared as discrete cytoplasmic staining in large cells from bone marrow of infected individuals. In contrast, bone marrow smears from normal controls displayed substantially less or no staining for iNOS compared with patients’ smears. Shown as a positive control for the staining procedure, the same bone marrow samples were stained for a protein that is irrelevant to this study but is expressed in both samples, IL-12 p35 (Fig. 7, C and D).

Human MDMs cultured with parasites and IFN-γ also stained positively for iNOS protein. Using the same infection protocol that resulted in l-NMMA inhibition of parasite killing (Fig. 5B), human MDMs were cultured on slides with IFN-γ with or without parasites and stained for iNOS. Staining of nuclei with the counterstain Nuclear Fast Red is evident in Fig. 7E. MDMs that had been exposed to parasites for 96 h and to IFN-γ for 48 h showed increased cytoplasmic staining for Ab to iNOS compared with those treated with IFN-γ alone (Fig. 7F). Together the RNase protection and immunohistochemistry data indicate that iNOS is expressed by human macrophages both in vitro and in vivo during L. chagasi infection.

Discussion

The purpose of this study was to document and compare the involvement of O₂⁻ and NO’ in leishmanicidal activity of murine and human macrophages. Our data indicate that O₂⁻ was generated in response to phagocytosis of promastigotes by both human and murine macrophages, but the amount generated by human macrophages was quantitatively greater. Although this difference could merely reflect differences in cell size, O₂⁻ scavenging affected leishmania survival more significantly in human than in murine macrophages. Superoxide generation was dependent on opsonization with serum-derived components, whereas opsonization did not influence the formation of NO’. Similar to published reports, we
detected nitrite reflecting NO’ production by murine macrophages, but neither nitrite nor nitrate was detected in human macrophages in response to leishmania phagocytosis (10). Nonetheless the biologic consequences of O2− and NO’ production by both murine and human macrophages were documented by the enhanced intracellular survival of parasites in cells in which their formation was specifically inhibited. Furthermore, we documented iNOS expression by human macrophages in vitro and in bone marrow samples from patients infected with L. chagasi. Thus, iNOS expression is not suppressed during human visceral leishmaniasis, and NO’ seems to contribute in some way toward intracellular killing of L. chagasi within human macrophages.

The kinetics of O2− vs NO’ production by macrophages in response to L. chagasi infection are different. Prior studies using luminol-enhanced chemiluminescence showed that a respiratory burst occurs within 1 h of initial phagocytosis of serum-opsonized L. donovani promastigotes by human MDMs (30). O2− has also been detected in murine peritoneal macrophages after phagocytosis of L. donovani (2). During the current study we detected O2− production and an effect of O2− scavenging early after promastigote phagocytosis, whereas the effect of NO’ occurred later (48–72 h after infection). These observations are consistent with a requirement for iNOS transcription and translation before NO’ generation. IFN-γ enhanced macrophage-mediated killing of leishmania by either murine or human cells (see Fig. 5B, 4 vs 48 h control samples), and this effect was dramatically reversed by inhibiting NO’-mediated parasite killing with L-NMMA. These observations are logical, in that IFN-γ is necessary for transcriptional activation of the iNOS promoter in both human and murine cells (33).

Macrophage infection with L. donovani amastigotes has been shown to have a profound effect on several key macrophage functions. The presence of intracellular L. donovani amastigotes leads to diminished PMA-stimulated respiratory burst activity, PKC translocation and activation, and mobilization of intracellular calcium stores (15, 16). During established amastigote infection, the expression of MHC class I and II Ags is down-modulated, as is the expression of the costimulatory molecule B7.2 (34). These events could interfere with the presentation of parasite Ags to T cells. Several sites on the NADPH oxidase p47^phox subunit are phosphorylated by protein kinase C (35), a necessary step before oxidase activation. Thus, amastigote infection is expected to impair both phagocytosis and the oxidative burst. Human macrophages containing L. donovani amastigotes are also unresponsive to IFN-γ stimulation due to impaired tyrosine phosphorylation of Jak1, Jak2, and the transcription factor Stat1 (17). Macrophage phosphatase activity and the expression of SHP-1 are increased (36, 37). Many of the signaling events that are attenuated by intracellular amastigote infection can also be mediated by treatment with isolated LPG from the promastigote surface membrane (18, 19, 38). LPG can also directly scavenge oxygen-derived radicals (39). However, because amastigotes express low to undetectable levels of LPG, this molecule probably could not explain all of the above observations (40). The extent to which LPG or other parasite molecules can partially or totally abrogate macrophage responses during initial infection with promastigotes or during established amastigote infection is not entirely clear.

Previous work demonstrated a role for NO’ in controlling infection of murine macrophages with Leishmania sp. (6, 8, 32, 41–44). IFN-γ-induced intracellular killing proceeds through the intermediate formation of TNF-α. The roles of both NO’ and O2− in murine L. donovani infection have been addressed by the use of the iNOS knockout and X-CGD mice. Mice lacking iNOS could not control infection, whereas mice lacking gp91^phox (X-CGD mice), and

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**FIGURE 7.** Immunohistochemical detection of iNOS in human bone marrow smears. Human bone marrow smears from a normal control (A and C) or a patient with visceral leishmaniasis (B and D) were tested for expression of iNOS (A and B) using immunohistochemistry. Arrows indicate cells staining positively for iNOS. Only nuclei stained with the counterstain (Nuclear Fast Red) are evident in the bone marrow smear from the control patient (A). Shown as a positive control for the staining procedure, many cells in both control and visceral leishmaniasis patient bone marrow smears stained positively for IL-12 p35 (C and D). Again, arrows show examples of cells staining positively in their cytoplasm. Micrographs are representative of four experiments (magnification, ×400). E and F, MDMs derived from normal human peripheral blood from separate individuals (magnification, ×400). E and F, MDMs derived from normal human peripheral blood were incubated without (E) or with (F) L. chagasi promastigotes followed by addition of IFN-γ, exactly as in Fig. 5B. Immunohistochemical staining for iNOS was performed. Control slides using secondary Ab alone were negative for all experiments (data not shown). Micrographs are representative of three experiments from separate individuals (magnification, ×500).
therefore unable to generate a respiratory burst, exhibited a delayed inflammatory response, but eventually controlled infection (45). The authors concluded that reactive nitrogen intermediates were sufficient to clear Leishmania infection in this model, although O$_2^-$ contributes to the efficiency of parasite clearance. Our data document the role of both oxidants in killing intracellular Leishmania in vitro, complementing these previous in vivo findings in murine models. NO$^-$ is an important signal transduction intermediate, and it is not clear whether the accelerated infection of knockout mice was due to defects in macrophage microbicidal activity or aberrant signaling (7).

It is of particular importance that Leishmania survival in human macrophages was enhanced by l-NMMA, because there has been considerable controversy over the importance of NO$^-$ in human macrophage microbicidal functions. Consistent with our results, other laboratories have also had difficulty detecting NO$^-$ in cultured human cells. This finding could be explained either by low levels of NO$^-$ produced or by efficient intracellular scavenging of the molecule before its release as NO$_2^-$. Nonetheless, our ability to detect a biological consequence of NO$^-$ formation, a finding that contrasts with some prior reports (10), supports this pathway as being important for the leishmanicidal activity of human cells. The difference between the results presented here and literature reports could be due to our use of different kinetics in infection studies or to differences in susceptibility of different Leishmania sp. to NO$^-$ killing. The presence of iNOS in human MDMs cultured in the presence of IFN-$\gamma$ and parasites enhances the functional data found with the NO$^-$ inhibitor l-NMMA.

Vouldoukis et al. reported that ligation of human macrophage CD23 (FceRII) with IgE elicited detectable NO$^-$ production in the presence of L. major or L. infantum and contributed to parasite killing, either independently or in the presence of IFN-$\gamma$ (11). This result could be explained by the action of iNOS or endothelial NOS, which is known to be activated by ligation of CD23 via up-regulation of CD11b and CD11c (46). Although we could not repeat the finding of NO$^-$ during L. chagasi infection of MDMs, we also found a biological effect of iNOS inhibition on parasite survival in the absence of CD23 ligation. As we did not use the same Leishmania sp. as Vouldoukis, our results cannot be directly compared. However, it is possible that the lack of effect of CD23 ligation in our system could in part be attributed to the probability that L. chagasi infection caused a loss of surface expression of CD23 on human macrophages, an observation made using B cells and a macrophage cell line (47).

Both iNOS mRNA and protein were expressed in bone marrow aspirates from Brazilians with symptomatic visceral leishmaniasis, and the level of iNOS mRNA was greater than that in normal controls. Consistent with our data, a prior report showed iNOS protein in a human splenic granuloma from a patient infected with L. donovani (48). Thus, despite the immunosuppression that occurs during human visceral leishmaniasis, the expression of this molecule with potential microbicidal activity was not suppressed. This observation probably reflects the balance between factors enhancing leishmanicidal activity (e.g., iNOS and IFN-$\gamma$) and factors inhibiting macrophage activation (e.g., TGF-$\beta$ and IL-10) during human infection. There is evidence in the literature that macrophage iNOS expression is also enhanced in the presence of other intracellular organisms. Importantly, Nicholson et al. previously demonstrated that iNOS is expressed in alveolar macrophages of human tuberculosis patients (12), as we report here for visceral leishmaniasis.

It is becoming clear that oxidants act not only as anti-microbial effectors, but also through effects on signaling and recruitment of inflammatory cells (49). Thus, NO$^-$ has been shown to be important not only for direct antimicrobial activity but also as a signaling molecule promoting IL-12- and IFN-$\alpha$/$\beta$-mediated activation of NK cells (7). The methods used in this study would not differentiate between a role of NO$^-$ in direct microbicidal activity vs a role as a signaling intermediate. The same applies to O$_2^-$, whose role in signaling has been documented in a few situations (50, 51). Furthermore, O$_2^-$ has been implicated as a sink for NO$, potentially explaining why it is not detected in human macrophages that produce more O$_2^-$ (44). The exact molecular mechanisms through which these radicals promote intracellular killing of leishmania have yet to be fully defined.

Our data support a model in which intracellular leishmania killing is effected initially through complement opsonization and stimulation of O$_2^-$ generation during the initial phagocytosis event in both murine and human macrophages. Once intracellular, the macrophage becomes quiescent, and amastigotes replicate. Late intracellular killing can occur through activation of macrophages by lymphocyte-derived factors, including IFN-$\gamma$, invoking a mechanism that requires NO$^-$ in both murine and human cells. Among other possibilities, the greater amount of NO$^-$ detected in supernatants of murine cells could reflect a greater importance of this effector molecule in leishmanicidal activity of murine as opposed to human cells. Nonetheless, our data support a model in which both human and murine macrophages use similar pathways, albeit to differing extents, to effect killing of this obligate intracellular parasite.

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