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Original Contribution

ROLE OF PEROXYNITRITE IN MACROPHAGE MICROBICIDAL MECHANISMS IN VIVO REVEALED BY PROTEIN NITRATION AND HYDROXYLATION

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Abstract—The cytotoxins produced by phagocytic cells lacking peroxidases such as macrophages remain elusive. To elucidate macrophage microbicidal mechanisms in vivo, we compared the lesion tissue responses of resistant (C57Bl/6) and susceptible (BALB/c) mice to *Leishmania amazonensis* infection. This comparison demonstrated that parasite control relied on lesion macrophage activation with inducible nitric oxide synthase expression (iNOS), nitric oxide synthesis, and extensive nitration of parasites inside macrophage phagolysosomes at an early infection stage. Nitration and iNOS expression were monitored by confocal microscopy; nitric oxide synthesis was monitored by EPR. The main macrophage nitrating agent was shown to be peroxynitrite derived because parasite nitration occurred in the virtual absence of polymorphonuclear cells (monitored as peroxidase activity) and was accompanied by protein hydroxylation (monitored as 3-hydroxytyrosine levels). In vitro studies confirmed that peroxynitrite is cytotoxic to parasites whereas nitric oxide is cytostatic. The results indicate that peroxynitrite is likely to be produced close to the parasites and most of it reacts with carbon dioxide to produce carbonate radical anion and nitrogen dioxide whose concerted action leads to parasite nitration. In parallel, some peroxynitrite decomposition to the hydroxyl radical should occur due to the detection of hydroxylated proteins in the healing tissues. Consequently, peroxynitrite and derived radicals are likely to be important macrophage-derived cytotoxins.

Keywords—Macrophage-derived cytotoxins, Peroxynitrite, Protein nitration and hydroxylation, Nitric oxide, Superoxide anion, Carbonate radical anion, Intracellular microorganisms, *Leishmania*, Free radicals

INTRODUCTION

The phagocytes of higher organisms have evolved to become remarkably effective in overcoming microbial infection. Although the respiratory-induced chemistry of these cells has been actively investigated for three decades, the identities of the toxins and their microbicidal mechanisms are not completely understood. The major oxidative microbicidal mechanism of peroxidase-containing phagocytic cells such as neutrophils is considered to rely on respiration-triggered formation of the highly cytotoxic compound hypochlorous acid ([1], and references therein). The cytotoxins produced by phagocytic cells lacking peroxidases but possessing active NADPH oxidases such as macrophages and myeloperoxidase-deficient neutrophils are more elusive. Superoxide anion, the product of NADPH oxidases, inactivates metabolically important [4Fe-4S] cluster-containing enzymes [2], but it is unlikely to be highly cytotoxic by itself [1]. Consequently, the discovery that activated macrophages express inducible nitric oxide synthase (iNOS) to produce copious amounts of nitric oxide from L-arginine [3,4] has provided new perspectives for the understanding of macrophage-mediated pathogen killing. Like superoxide anion, nitric oxide is relatively unreactive toward biomolecules, but, being free radicals, superoxide anion and nitric oxide combine at diffusion-controlled

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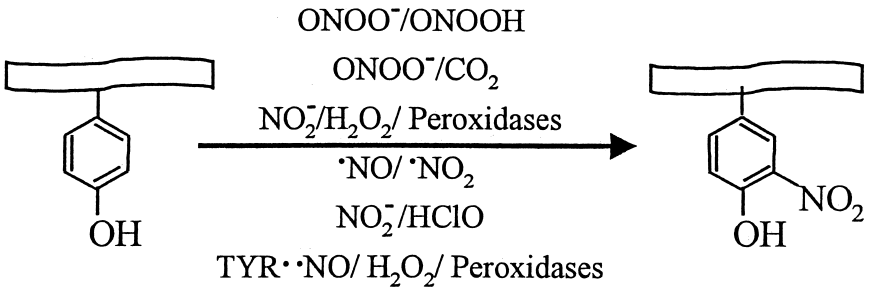

Fig. 1. Schematic representation of the systems that can be responsible for nitration of protein-tyrosine residues in vivo [6,7,16–19].

rates to produce peroxynitrite [5]. Since peroxynitrite oxidizes and nitrates a variety of biological targets it has received much attention as the potential mediator of nitric oxide cytotoxic effects.

A pathogenic role for peroxynitrite has been investigated in various human diseases, particularly in neurodegenerative and cardiovascular disorders ([6], and references therein). Contributions of peroxynitrite to microorganism killing have also been proposed [1,7–12], but in vivo studies were limited to murine leishmanial [13] and mycoplasmal [14] infections. The role of peroxynitrite, either as a mediator of human diseases or as a toxin against invading microorganisms, has been mostly inferred from the detection of 3-nitrotyrosine residues in proteins of injured tissues or cells [6,15]. Initially, nitration of tyrosine residues under physiological conditions was considered to be dependent on peroxynitrite formation. More recently, however, other mechanisms have been demonstrated to be possible such as oxidation of nitrite by hypochlorous acid and by peroxidase enzymes [16–19] (see, Fig. 1). Certainly, additional experimental approaches are required to unravel the cytotoxic mechanisms prevailing in various pathophysiological conditions. Here, we explore the advantages of two murine models of *L. amazonensis* infection to elucidate macrophage microbicidal mechanisms in vivo.

Leishmania, an obligate intramacrophage protozoon, produces a wide spectrum of disease in humans and mice. Murine experimental leishmaniasis has emerged as a useful model not only for the understanding of human leishmaniasis but also of other diseases caused by intracellular pathogens such as tuberculosis, listeriosis, and toxoplasmosis. Strains of mice such as C57Bl/6 resist cutaneous *Leishmania* infection with clinical, albeit not parasitological, cure within a few weeks. By contrast, BALB/c mice and other strains fail to control parasite multiplication whose dissemination to visceral organs results in host death. Resistance and susceptibility are generally related to the ability of the host to set up T cell responses of the Th1 or Th2 type, respectively ([20], and references therein). These responses have been associated with the production or nonproduction of nitric oxide by the infected macrophages, and, indeed, a role for nitric oxide in host defense against pathogens was first demonstrated in the murine *Leishmania* model [21–23]. The responses of C57BL/6 and BALB/c mice to *L. amazonensis* represent extremes in the response to the parasite, but in both cases a macrophage infiltrate predominates in the cutaneous lesions [24]. Thus, the models are useful to compare production of nitric oxide and derived oxidants by the same cell (macrophage) challenged by the same stimulus (parasite), but responding in opposite directions (controlled/uncontrolled infection). With this comparison, we provide here multiple lines of evidence for the role of peroxynitrite and derived radicals as predominant microbicidal agents produced from macrophages in vivo.

MATERIALS AND METHODS

Parasite and infection

L. amazonensis (MHOM/BR/73/M2269) amastigotes were obtained from footpad lesions of BALB/c mice as previously described [13]. BALB/c and C57Bl/6 mice were injected subcutaneously in the right hind footpad with 2×10^6 amastigotes at 6 weeks of age. Female animals were from the Animal Facilities of Universidade Estadual de Campinas. The course of infection was monitored by measuring the increase in footpad thickness compared with the contralateral uninfected footpad with a dial caliper. At designed periods, mice were sacrificed to estimate parasite burdens in the footpad by a limiting-dilution procedure [25].

Electron paramagnetic resonance (EPR) measurements

Boneless footpads from mice were extruded with a syringe into quartz tubes (4.2 mm i.d. $\times$ 5.0 mm o.d.) and frozen in liquid nitrogen [13]. The EPR spectra were obtained with a Bruker ER 200 D-SRC spectrometer using a fingertip liquid nitrogen dewar (77 K). Spectrom-

eter conditions were as follow: microwave power, 10 mW; modulation amplitude, 0.5 mT; time constant, 1 s; scan rate, 0.12 mT/s. The data were fed to IBM/AT computer where baseline subtraction and double integration were performed. The concentration of nitrosyl complexes was estimated by double integration of EPR spectra using known concentrations of hemoglobin-nitrosyl complexes as standard [13].

Confocal immunofluorescence microscopy

Paraffin-embedded footpad sections were obtained as previously described [13] and stained with rabbit polyclonal anti-iNOS primary antibody and developed with FITC-conjugated antirabbit IgG. Subsequently, they were treated with mouse monoclonal 3-nitrotyrosine primary antibody and developed with Cy3-conjugated antimouse IgG. Blocking with 3-nitrotyrosine was used to verify specificity. DNA was labeled with DAPI. Before staining, some sections were microwave stimulated [26], but no major immunoreactivity differences were observed. Sections were examined as previously described [27] on a BioRad (Hercules, CA, USA) 1024-UV confocal system attached to a Zeiss (Thornwood, NY, USA) Axiovert 100 microscope. Rabbit polyclonal anti-iNOS antibody, FITC-conjugated antirabbit IgG, Cy3-conjugated antimouse antibody, and Cy3-conjugated antirabbit antibody were from Sigma Chemical Co. (St. Louis, MO, USA). Mouse monoclonal anti-3-nitrotyrosine antibody was provided by J. S. Beckman.

Peroxidase activity

Tissue from footpads was homogenized in 2.5 volumes of 50 mM TRIS buffer, pH 7.4, containing 0.5% Triton X-100, 5mM EDTA, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 1 mM PMSF, and 1 mM sodium orthovanadate. After centrifugation (10,000 $\times$ *g*) for 10 min at 4°C, an aliquot of the supernatant was used for protein determination by the Bradford method and another for peroxidase assay. This activity was quantified as previously described [28], with minor modifications. An aliquot of the diluted supernatant (about 5 μg/ml protein) was added to 50 mM phosphate buffer, pH 6.2, containing 0.8 mM *o*-dianisidine and 1.5 mM hydrogen peroxide, and the rate of substrate oxidation was monitored spectrophotometrically at 460 nm in 1.0 cm light path cuvettes. One unit of peroxidase activity was defined as the amount of enzyme necessary to produce one micromole of oxidized *o*-dianisidine ($\epsilon_{460} = 1.13 \times 10^4$ M^{-1} cm^{-1}) per minute at 25°C.

DOPA analysis

Tissue from footpads was homogenized in 3 volumes of 10 mM acetate buffer, pH 6.5, containing 0.5% Triton X-100 and 100 μM desferrioxamine. After centrifugation (11,000 $\times$ *g*) for 10 min at 4°C, an aliquot of the supernatants was treated with pronase (0.33 mg/ml) for 18 h at 50°C [29]. Subsequently, the samples were treated with 2 volumes of 10% trichloroacetic acid and centrifuged (11,000 $\times$ *g*) for 10 min at 4°C. The supernatants were filtered and analyzed by HPLC to determine tyrosine and DOPA content. The HPLC system consisted of a Waters Associates (Milford, MA, USA) model 625LC apparatus equipped with an ESA, Inc. (Chelmsford, MA, USA) electrochemical detector (glassy carbon electrode) with applied potentials of 0.24 and 0.65 V for DOPA and tyrosine detection, respectively. Chromatographic separation was carried out using a Supelcosil LC-18S (4.5 $\times$ 250 mm, 5 μm particle size) column and 50 mM sodium acetate plus 50 mM sodium citrate, pH 3.1, containing 8% methanol as the mobile phase (flow 0.6 ml/min). DOPA and tyrosine were identified and quantified by comparison with known concentrations of standards run under the same chromatographic conditions. DOPA level was expressed in relation to tyrosine level in the same sample.

Leishmanicidal activity in vitro

Axenic amastigotes were obtained by adaptation of *L. amazonensis* promastigotes recently transformed from lesion-derived amastigotes as previously described [30]. Amastigote-like parasites were maintained at 32°C in Scheneider's Drosophila medium, pH 4.6, supplemented with 20% fetal calf serum and gentamicin (24 μg/ml). Their infectivity was verified by subcutaneous injection into the footpad of BALB/c mice. Peroxynitrite was synthesized and analyzed as previously described [5,31]. The nitric oxide donor used was NOC-5 (1-hydroxy-2-oxo-3 (3-aminopropyl)-3-isopropyl-1-triazene) obtained from Alexis Corp. (San Diego, CA, USA). Stock solutions of NOC-5 were prepared in 0.01 M NaOH. Before in vitro treatment, the parasites were harvested by centrifugation, washed twice with phosphate-buffered saline, and resuspended in 200 mM phosphate buffer, pH 7.4. The parasites in 96-well plates (Costar, Cambridge, MA, USA) (4×10^6 cells/well) were treated with peroxynitrite (for 10 min) or NOC-5 (for 30 min) in 200 mM phosphate buffer, pH 7.4 (120 μl final volume). Treatment times were selected to guarantee more than 95% decomposition of the used concentrations of both peroxynitrite ($t_{1/2} < 1$ s) and NOC-5 ($t_{1/2} = 360$ s). Since stock solutions of peroxynitrite and NOC-5 were alkaline, the volume of NaOH corresponding to 2 mM con-

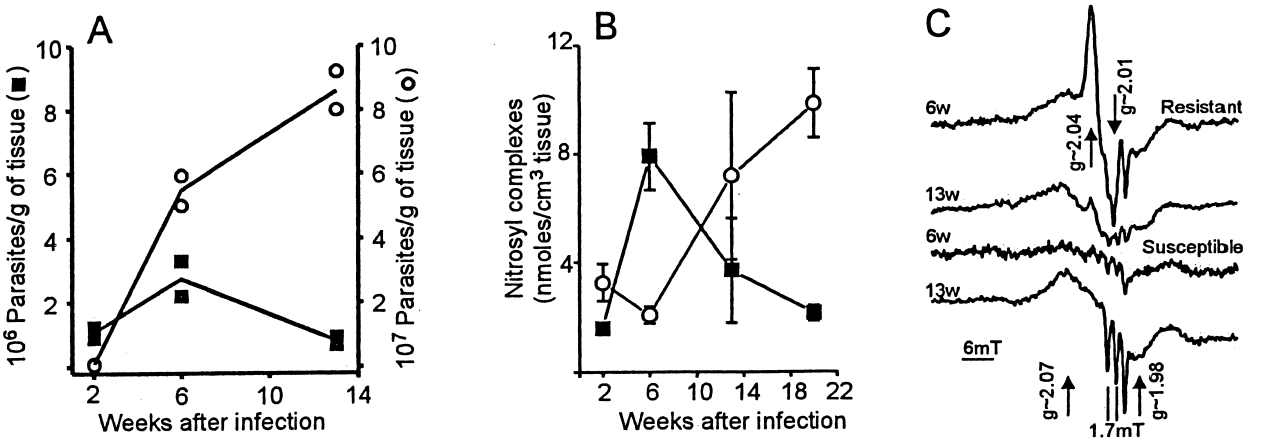

Fig. 2. Course of *L. amazonensis* infection in resistant (C57Bl/6) (■) and susceptible (BALB/c) mice (○) monitored by changes in some footpad lesion parameters. (A) Parasite numbers. The values represent the mean from two mice per group. (B) Nitric oxide synthesis monitored by low-temperature EPR as nitrosyl complex levels. The values represent the mean ± the standard error from at least three mice per group. (C) Representative EPR spectra obtained from the animal footpads where some shown *g* values characterize the iron-dithiol-dinitrosyl and heme-nitrosyl complexes detected in resistant and susceptible mice, respectively. Analyses were performed as described in Materials and Methods.

centration of peroxynitrite or NOC-5 was added to all parasite suspensions including the controls. After treatment, 200 μl of DME medium supplemented with 20% fetal calf serum, pH 7.4, was added. Subsequently, parasite cultures were maintained at 26°C and cell viability was determined from hemocytometer counts after erythrosin-B staining [32] at 1, 24, and 72 h.

RESULTS

Infection and parasite control

In resistant (C57Bl/6) mice injected into the footpad with 2×10^6 *L. amazonensis* amastigotes the skin lesion remained controlled [13,20]. Infection control was shown to correlate with systemic levels of nitric oxide monitored by EPR as hemoglobin nitrosyl complexes present in mouse blood [13]. Here, we examined localized changes that led to parasite restraint in the lesions. Maximum parasite numbers in footpad tissues occurred at about 6 weeks (Fig. 2A) concomitant with maximum nitric oxide synthesis, which was monitored by direct EPR as iron-dithiol-dinitrosyl complex levels (Figs. 2B and 2C). These complexes are known to be produced by the interaction of nitric oxide with cellular targets containing iron-sulfur centers. The in vivo targets remain to be fully characterized but the same EPR signal has been detected in cultures of macrophages expressing iNOS [33]. In agreement, nitric oxide detected in the footpads of resistant mice was enzymatically synthesized because a high expression of iNOS was detectable by confocal microscopy at 6 weeks after infection (Figs. 3A and 3B; green staining). Relevantly, iNOS was localized close to the limits of infected phagolysosomes. In addition, maximum nitric oxide synthesis and iNOS expression were paralleled by extensive protein nitration of the parasite inside the phagolysosomes, as clearly visualized by confocal microscopy (Figs. 3A and 3B; red staining). Parasite numbers decreased thereafter, showing that infection was controlled by the host. In contrast, susceptible mice presented marginal nitric oxide synthesis (Figs. 2B and 2C), iNOS expression, and parasite nitration (Figs. 3D and 3E) up to 6 weeks of infection. In parallel, parasite proliferation remained uncontrolled (Fig. 2A). This comparison suggests that nitration of parasite proteins is related to parasite control by resistant mice.

Late infection

Resistant mice are known to achieve clinical but not parasitological cure because a few parasites are detectable throughout their lifetime [20,34]. Accordingly, all infection parameters were considerably decreased by week 13 and longer, but they remained measurable (Figs. 2 and 4). Continued, even if relatively small, nitric oxide synthesis is likely to be required for sustained control of remaining parasites [34]. In contrast, infection progression in susceptible mice led to a continuous increase in parasite numbers (Fig. 2A) and late nitric oxide synthesis (Figs. 2B and 2C), iNOS expression, and nitration of parasites and host tissues (Fig. 4C and 4D). At week 13, most heavily parasitized macrophages became disrupted, impairing iNOS localization (Figs. 4C and 4D). At these stages, the presence of bacteria [13] and polymorphonuclear cells (data not shown) in the cutaneous lesions of

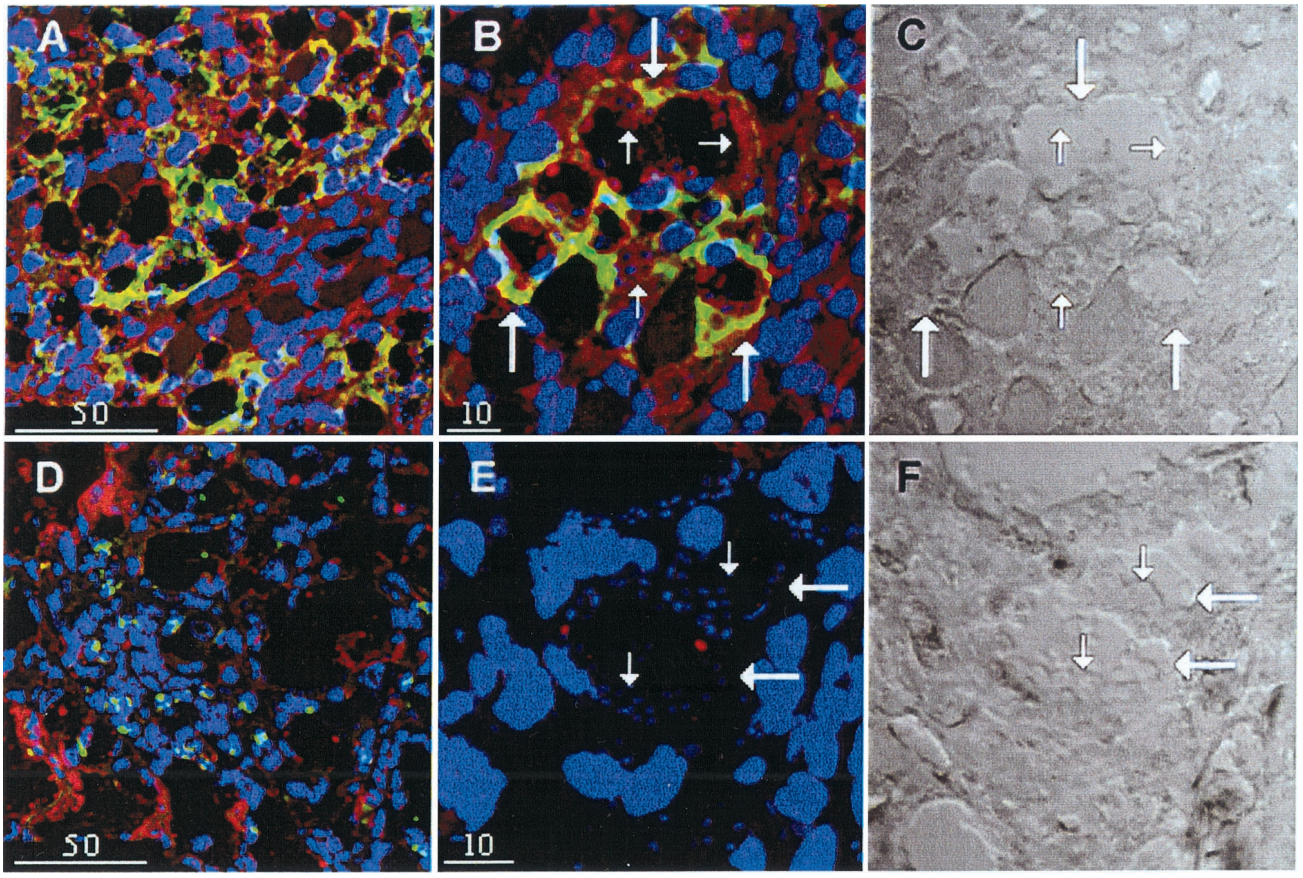

Fig. 3. Representative confocal microscopy images of footpad sections of resistant (A–C) and susceptible (D–F) mice at 6 weeks of infection. DNA (blue staining), iNOS (green staining), 3-nitrotyrosine residues (red staining), and colocalization of iNOS and 3-nitrotyrosine residues (yellow staining) were developed as described in Materials and Methods. Footpad sections of both mice strains showing general (A, D) and close (B, E) views; B and E were also visualized by Nomarski differential interference contrast (C, F). In the amplified views, phagolysosomes (big arrows) containing parasites (small arrows) either nitrated (B) or non-nitrated (blue DNA staining) (E) were evident. Magnification bars in micra.

susceptible mice was evident. Likewise, peroxidase activity was increased (Fig. 5A). Both macrophages and polymorphonuclear cells can be responsible for this late nitric oxide synthesis because at least some neutrophils express iNOS [35].

Noteworthy, nitric oxide generated by iNOS was detectable as heme nitrosyl complexes [36,37] rather than as iron-dithiol-dinitrosyl complexes, which are characteristic of activated macrophages [33] and were detected in resistant mice (Fig. 2C). Heme nitrosyl complexes may be revealing the presence of peroxidase-containing polymorphonuclear cells (Fig. 5A) or hemoglobin extraverted in the cutaneous lesions. Consequently, nitric oxide synthesis and parasite nitration observed in susceptible mice lesions are late events that occur when parasite burden is enormous (Fig. 2A, note the different scales; Figs. 4C and 4D). These events are likely to result from secondary bacterial infection [13] and may be independent of macrophage activation because NO was detectable mainly as heme nitrosyl rather than as iron-dithiol-dinitrosyl complexes (Fig. 2C). These late events are extremely useful for comparative purposes (Figs. 2 and 5), but their detailed discussion is beyond the scope of the present work that focuses on parasite control.

Peroxynitrite as the nitrating agent in resistant mice

Parasite numbers in the footpad of resistant mice were markedly decreased after nitration of parasite proteins (Figs. 2A and 3), showing the importance of establishing the nature of the nitrating agent. The nitration mechanisms proposed to be relevant under physiological conditions are dependent on peroxidase-catalyzed reactions, auto-oxidation of nitric oxide to nitrogen dioxide, and peroxynitrite-promoted processes (Fig. 1) [6,15–19]. Polymorphonuclear cells were barely detectable by optical microscopy in the lesions of resistant mice up to 13 weeks of infection (data not shown) (see, also [24]). In addition, peroxidase activity in resistant mouse footpads remained relatively constant at different stages of infec-

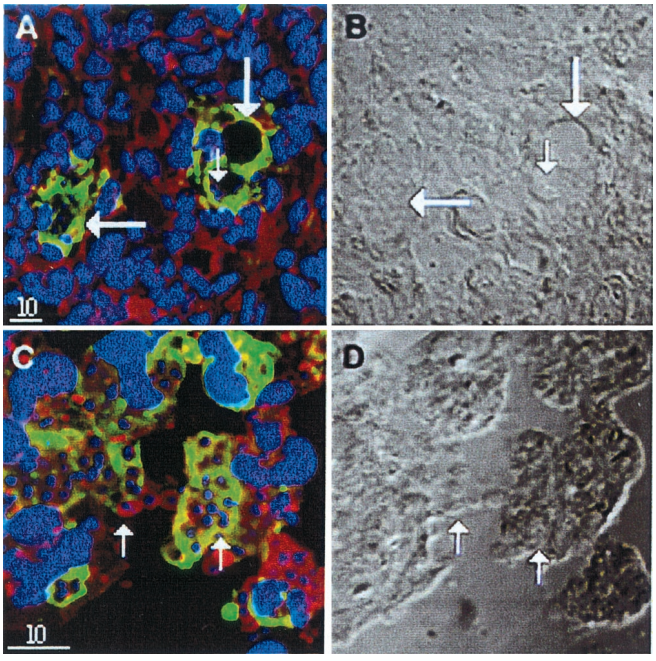

Fig. 4. Confocal microscopy images of footpad sections of resistant (A, B) and susceptible (C, D) mice at 13 weeks of infection. DNA (blue staining), iNOS (green staining), 3-nitrotyrosine residues (red staining), and colocalization of i-NOS and 3-nitrotyrosine residues (yellow staining) were developed as described in Materials and Methods. Phagolysosomes and parasites are indicated by big and small arrows, respectively. At this time, infected phagolysosomes and the corresponding iNOS staining are hardly found in resistant mice footpad sections and the figures (A, B) show one of these few regions. In contrast, the susceptible mice figures (C, D) are representative of the sections that show many parasites and extensive iNOS expression. Magnification bars in micra.

tion and was 10 times lower than in susceptible mice (Fig. 5A) that presented polymorphonuclear cells at late infection times (see above). This comparison argues against a peroxidase-mediated nitration mechanism for parasite control.

Equally unlikely is a mechanism dependent on nitric oxide production from nitric oxide auto-oxidation because nitric oxide alone is not an efficient nitrating agent at the pH of macrophage phagolysosomes [38] whose value is around 5.0 (4.7–5.2) [39]. In contrast, peroxynitrite ($ONOOH/ONOO^-$; ($pKa = 6.6$)) is capable to nitrate tyrosine residues at these pHs in a process that is increased by CO_2 [31,40–43], whose concentration is expected to be high in activated macrophages [1]. At acid pHs in particular, peroxynitrous acid is also able to hydroxylate tyrosine residues to produce DOPA [29,31]. Consequently, parallel production of 3-nitrotyrosine and DOPA was proposed as a useful tool to differentiate peroxynitrite from other nitrating species [31]. Here, the presence of DOPA in hydrolysates of proteins extracted from the footpads of resistant and susceptible mice was examined by HPLC. DOPA was easily detectable at 6 weeks of infection and its levels were found to be 63.8 ± 17.1 and 23.3 ± 7.0 mmol DOPA/mol tyrosine for resistant and susceptible mice, respectively (Fig. 5B). Noninfected animals of both strains presented undetectable DOPA levels (< 1 mmol/mol tyrosine under our experimental conditions). Interestingly, the higher DOPA levels in the lesions of resistant mice (Fig. 5B) correlated with higher nitric oxide synthesis (Figs. 2B and 2C) and parasite nitration levels (Fig. 3) but with lower peroxidase activity (Fig. 5A). Taken together, these results strongly support peroxynitrite as the nitrating agent at week 6, which is crucial for parasite control by resistant mice.

Peroxynitrite-mediated amastigote killing in vitro

Amastigotes are the parasite forms that live in macrophages. In culture, these forms can be maintained as amastigote-like parasites by differentiation of the promastigote forms [30]. The in vitro susceptibility of amastigote-like parasite to peroxynitrite and a nitric oxide donor was compared (Fig. 6). Cell viability was affected by peroxynitrite in a concentration-dependent manner. After 24 h, the surviving cells started to proliferate (Fig. 6A), a process that was partially dependent on back differentiation to the promastigote form (data not shown). In contrast, cell viability was marginally affected by nitric oxide but cell proliferation was constrained (Fig. 6B). The cytotoxic effect of peroxynitrite compared with the cytostatic effect of NO in vitro supports peroxynitrite as the macrophage effector of *Leishmania* killing in vivo.

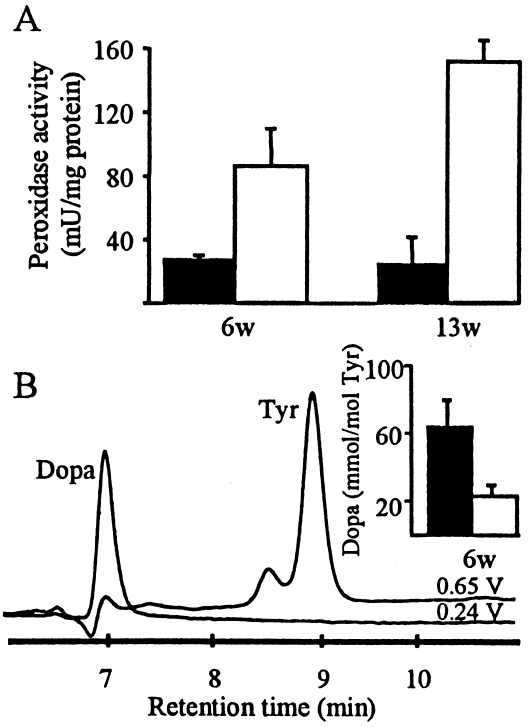

Fig. 5. Peroxidase activity (A) and DOPA levels (B) determined in footpad tissues of resistant (closed bars) and susceptible (open bars) mice at the specified infection times. (B) Representative chromatograms of hydrolysates of lesion proteins from resistant mice detected at 0.24 V (50 nA, full scale) and 0.65 V (500 nA, full scale) show the presence of DOPA and tyrosine (Tyr), respectively. The inset shows DOPA levels determined for resistant and susceptible mice at 6 weeks of infection; DOPA levels in uninfected mice of both strains were marginal ($<$ 1 mmol/mol tyrosine under the used experimental conditions). Analyses were performed as described in Materials and Methods; the values represent the mean $\pm$ the standard error from at least three mice per group.

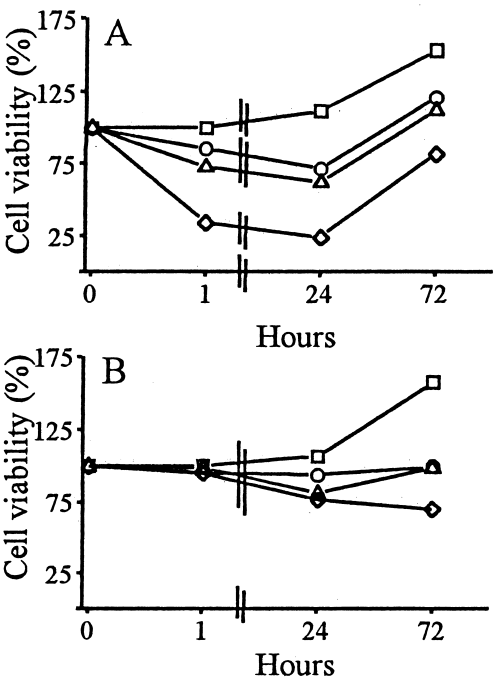

Fig. 6. Effects of peroxynitrite (A) and nitric oxide released from NOC-5 (B) on the viability of amastigote-like forms of *L. amazonensis*. The used concentrations of peroxynitrite were: 0 (□), 0.5 mM (○), 1 mM (Δ), and 2 mM (◇). Experimental procedures were as described in Materials and Methods. The values are a percentage of the values of untreated parasites at zero time and were expressed as the mean from two parallel experiments. Additional independent experiments provided the same profiles as those shown in the figure. Cell viability of parasites treated with decomposed peroxynitrite or NOC-5 matched that of untreated amastigotes.

DISCUSSION

Control of *L. amazonensis* infection provided an adequate model to distinguish peroxynitrite from other microbicidal agents that can be produced by activated macrophages in vivo. Particularly important was the possibility of comparing the responses of resistant and susceptible mice to establish the factors relevant for parasite control (Figs. 2–5). For instance, polymorphonuclear cell migration and associated peroxidase activity appeared irrelevant because they were more pronounced in susceptible than in resistant mice (Fig. 5A). In contrast, DOPA levels were higher in footpad proteins from resistant than susceptible mice (Fig. 5B) whose large cutaneous lesions containing huge parasite numbers (Figs. 2 and 3) provided a satisfactory control for eventual ex vivo DOPA production. Comparison of infection parameters in resistant and susceptible mice demonstrated that infection control relied on lesion macrophage activation with iNOS expression, nitric oxide production, and extensive nitration of parasites inside macrophage phagolysosomes at an early infection stage as accomplished by the resistant mice (Figs. 2 and 3; see, also, Results). In this case, parasite numbers decreased dramatically after nitration of their proteins (Figs. 2 and 3), indicating that the process is likely to be relevant for parasite elimination. The main macrophage-derived nitrating agent was shown to be derived from peroxynitrite because parasite nitration (Figs. 3A and 3B) occurred at low pH [38,39] in a peroxidase-deprived environment (Fig. 5A) and was accompanied by the production of DOPA in the healing lesions (Fig. 5B) [31].

Relevantly, iNOS was visualized by confocal microscopy close to the limits of the phagolysosomes (Fig. 3B) and such spatial confinement was adequate for peroxynitrite production adjacent to the parasite because of the localization of the superoxide anion producer, NADPH oxidase, within the phagolysosome membrane. Production of peroxynitrite close to the parasite is important due to the intrinsic reactivity of the compound that otherwise could be scavenged by other biotargets present in phagolysosomes. However, the fast reaction of peroxyni-

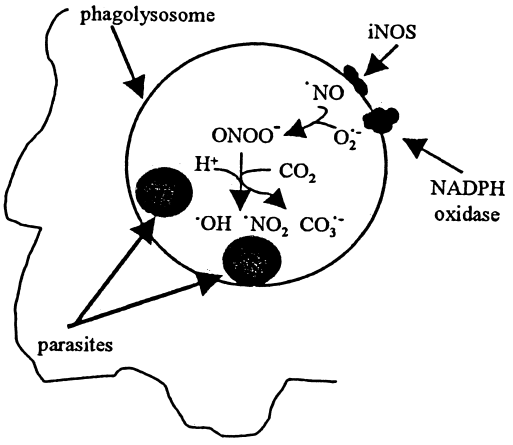

Fig. 7. Schematic representation of the production of macrophage-derived cytotoxins proposed to be relevant for *Leishmania* control in whole mammalian hosts. This proposition was based on the described results being particularly relevant to compare the scheme with the data shown in Fig. 3B.

trite with carbon dioxide [44], whose concentration is expected to be high in activated macrophages [1], appears to predominate because of the extensive nitration of parasite proteins. Indeed, the reaction of peroxynitrite with carbon dioxide produces carbonate radical anion and nitrogen dioxide [45,46], whose concerted action leads to efficient nitration of tyrosine residues [31,40–43]. In parallel, some decomposition of peroxynitrous acid to hydroxyl radical and nitrogen dioxide occurs as expected [31], and proved by detection of increased DOPA levels in proteins from the lesions (Figs. 4B and 7). Taken together, these results indicate that peroxynitrite and derived radicals are the predominant leishmanicidal agents produced from macrophages in vivo (Fig. 7). Relevantly, recent studies of visceral *L. donovani* infection in both iNOS knockout and chronic granulomatous disease mice, indicated that reactive oxygen- and nitrogen-derived species act together to regulate the initial extent of parasite replication [47].

The results described here can not provide detailed information about the mechanisms by which peroxynitrite is cytotoxic (Fig. 6), but the in vivo results (Figs. 2 and 3) suggest that nitration/oxidation of parasite membrane proteins may be an important event. Functional alteration of membrane proteins may impair intracellular ionic composition and transport of essential metabolites, all of which are processes crucial to cell survival. This is particularly relevant to *Leishmania* that possesses a proton-translocating ATPase in the plasma membrane to maintain its internal pH around neutrality in spite of the acidic phagolysosome pH [48].

In conclusion, our results indicate that parallel protein nitration and hydroxylation [31] can, indeed, be useful to distinguish peroxynitrite from other in vivo nitrating agents and the approach should be tested in other models. Moreover, the results demonstrate that peroxynitrite and its derived radicals, carbonate radical anion, nitrogen dioxide, and hydroxyl radicals, are important leishmanicidal agents in whole mammalian hosts (Fig. 7). Consequently, these species are likely to be the main cytotoxins produced by macrophages in vivo.

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ABBREVIATIONS

DAPI—4′,6-diamidino-2-phenylindole

DOPA—3-hydroxytyrosine, 3,4-dihydroxyphenylalanine

iNOs—inducible nitric oxide synthase

NOC 5—1-hydroxy-2oxo-3(3-aminopropyl)-3-isopropyl-1-triazene

peroxynitrite—the sum of peroxynitrite anion ($ONOO^-$, oxoperoxonitrate (1-)) and peroxynitrous acid (ONOOH, hydrogen oxoperoxynitrate), unless specified