Photochemistry and Photobiology, 1996, 63(6): 750–754

Symposium-in-Print

Formation of Nitrosyl Hemoglobin and Nitrotyrosine during Murine Leishmaniasis*

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Received 24 October 1995; accepted 9 January 1996

ABSTRACT

Peroxynitrite, the potent oxidant formed by the fast reaction between nitric oxide and superoxide anion, has been suggested to be the reactive intermediate responsible for some of the pathologies associated with an overproduction of nitric oxide. In this report, we demonstrate that both nitric oxide and peroxynitrite are formed during infection of the susceptible mouse strain, BALB/c, with *Leishmania amazonensis*. Nitric oxide was detected as the nitrosyl hemoglobin complex by EPR analysis of blood drawn from mice at 35, 64 and 148 days of infection. The levels of nitrosyl hemoglobin complex increased with disease evolution, which in the murine model used is characterized by skin lesions, ulceration and visceralization of the parasites. Peroxynitrite formation was inferred from immunoreaction of homogenates obtained from footpad lesions in the late stages of the infection with anti-nitrotyrosine antibody; homogenates from parasites drawn from the lesions were also immunoreactive, although to a lesser extent. Analysis of protein homogenates by gel electrophoresis and western blots suggests that peroxynitrite may degrade proteins *in vivo*, in addition to nitrating them. The results demonstrate that peroxynitrite is formed during murine leishmaniasis and may play a role in the aggravation of the disease.

INTRODUCTION

Nitric oxide is an endogenously synthesized free radical that has been implicated in various physiological functions including blood pressure control, neurotransmission and immune regulation (1–3). Activated macrophages produce nitric oxide through oxidation of the amino acid L-arginine by an inducible nitric oxide synthase (4). Because inhibitors of this enzyme reduce the cytotoxicity of macrophages against bacteria and tumor cells, nitric oxide is considered to be a key weapon of activated macrophages against various invading cells (4,5). The precise microbicidal mechanisms, however, remain incompletely understood (6).

Nitric oxide itself can be cytotoxic through interaction with iron–sulfur clusters and heme groups of several metalloenzymes involved in cell energy metabolism (7) and DNA synthesis (8). The reactivity of nitric oxide against biological targets, however, can be greatly increased after its reaction with either oxygen, to produce nitrogen dioxide (Eq. 1), or with superoxide anion, to form peroxynitrite‡ (Eq. 2).

$$2\,{}^{\cdot}NO + O_2 \xrightarrow{k = 8 \times 10^6\ M^{-2}\ s^{-1}} 2\,{}^{\cdot}NO_2 \qquad (1)$$

$${}^{\cdot}NO + O_2^{\cdot-} \xrightarrow{k = 6.7 \times 10^9\ M^{-1}\ s^{-1}} ONOO^- \qquad (2)$$

$$ONOO^- + H^+ \underset{\longleftarrow}{\xrightarrow{pK_a = 6.8}} ONOOH \qquad (3)$$

The formation of nitrogen dioxide through Eq. 1 is dependent on the square of nitric oxide concentration and is expected to be slow under biologically relevant conditions (9,10). By contrast, peroxynitrite production is a likely possibility due to the almost diffusion-controlled rate constant of the reaction between superoxide anion and nitric oxide (11) (Eq. 2), species that are formed by different cell types, including macrophages, neutrophils and Kupffer cells as has been previously discussed (12,13).

Peroxynitrite is highly reactive at physiological pH because it protonates to peroxynitrous acid (Eq. 3), which can oxidize a variety of biomolecules by one- and two-electron processes (12–15). The former characterizes the so-called hydroxyl radical-like reactivity of the oxidant, a pathway that also produces the reactive nitrogen dioxide radical. Peroxynitrite is able to nitrate aromatic amino acids (16,17), and this process has been explored to develop antibodies that recognize protein nitrotyrosines. Nitrotyrosine formation can thus be used as markers of peroxynitrite formation *in vivo* (18,19). Because peroxynitrite is highly reactive (12–15) and

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‡Herein, the term peroxynitrite is used to refer to the sum of all possible forms of peroxynitrite anion and peroxynitrous acid unless otherwise specified.

toxic to cells (20) and microorganisms (10, 21–23), it has been suggested to be the oxidant responsible for several of the pathological conditions associated with an overproduction of nitric oxide (12–15).

In the last 2 years, an important series of papers have presented evidence for peroxynitrite formation *in vivo,* particularly during inflammatory processes. For instance, increased nitrotyrosine levels have been detected in atherosclerotic plaques of human coronary vessels (18), in the lung of infants with acute lung injury (19) and in synovial fluid and plasma from patients with rheumatoid arthritis (24). In animal models, an increase in nitrotyrosine immunoreactivity was found in lung macrophages (25) and in the aorta of rats injected with bacterial endotoxin (26). To the best of our knowledge, however, formation of peroxynitrite during parasitic infections has never been reported in the literature.

In this study, we evaluate the production of both, nitric oxide and peroxynitrite during infection of BALB/c mice with *Leishmania amazonensis.* This murine model appears to be particularly promising for study because it develops uncontrolled cutaneous lesions infiltrated with a mononuclear phagocyte cell population composed of resident dermal macrophages and newly recruited monocytes (27,28). Although an increase in the macrophage pool is usually triggered by protective immune mechanisms of the host, in *Leishmania* infection this process provides additional cells for invading parasites.

MATERIALS AND METHODS

Peroxynitrite. Peroxynitrite was synthesized in a quenched flow reactor as previously described (22,23,29). Peroxynitrite stock solutions were prepared with water to keep the pH above 13; their concentrations were determined in sodium hydroxide (0.1 *N*) by absorbance at 302 nm (ϵ = 1670 M^{-1} cm^{-1}).

Parasite and infection. Leishmania amazonensis (MHOM/BR/73/M2269) amastigotes were obtained from footpad lesions of BALB/c mice as previously described (30). Female BALB/c mice were injected subcutaneously into the right hind footpad with 2×10^6 amastigotes and footpad thickness was measured periodically with a dial caliper.

Protein homogenates. Footpads from infected (87–90 days after *L. amazonensis* inoculation) and normal mice were surgically removed, the tissues minced, resuspended and homogenized in phosphate buffer, pH 7.0, containing sodium chloride (0.15 m*M*), ethylenediaminetetraacetic acid (EDTA)§ (5 m*M*), benzamidine (1 m*M*), sodium bisulfite (5 m*M*), phenylthiocarbamide (0.5 m*M*) and *N*-α-*p*-tosyl-L-lysine chloromethyl-ketone (1 m*M*). The suspensions were filtered and centrifuged at 11 000 ***g*** for 5 min at 4°C. The supernatants were pooled and used in the immunoblot assays. Parasite homogenates were obtained by the above procedure after isolation from footpad lesions (30). Protein concentration was measured by the Coomassie blue method (31) using bovine serum albumin as standard. The homogenates were divided into aliquots and stored frozen till use.

Dot blot. Protein homogenates were dotted onto nitrocellulose filters (Bio-Rad Laboratories, Richmond, CA, USA) with a slot blot apparatus (Hybri-Slot™, BRL-Life Technologies Inc, Gaithersburg, MD, USA). After blotting, filters were incubated (32) with rabbit polyclonal immunoglobulin G (IgG) antibody directed against nitrotyrosine (18) (dilution 1:100) kindly provided by Dr. Joseph S. Beckman, University of Alabama, Birmingham. The secondary antibody consisted of peroxidase-conjugated goat anti-rabbit IgG (A6154, Sigma Chemical Co., St, Louis, MO, USA); development was performed with 3,3-diaminobenzidine (30). To confirm the specificity of the binding, two negative controls were employed, coincubation with 10 m*M* nitrotyrosine (18) and replacement of the primary antibody with rabbit preimmune serum. As positive controls, we used homogenates (1 mg protein/mL) pretreated with 1 m*M* peroxynitrite.

Immunoreaction was quantitated by densitometric scanning on a dual-wavelength flying-spot scanner (Shimadzu DZV, CS-9000-FDU-3).

Polyacrylamide gel electrophoresis and western blot. Protein homogenates (25 μg/lane) were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with 7–17% polyacrylamide gel slabs as previously described (33). Electrophoresis was performed at a constant voltage (25 V/cm) until the tracking dye (bromophenol blue) reached the bottom of the gels. These were stained with Coomassie blue R 0.2% (wt/vol) in acetic acid : methanol : water (10:50:40, vol/vol/vol) for proteins and destained with acetic acid : ethanol : water (8:25:75, vol/vol/vol) at room temperature. Protein molecular weight standards were purchased from Bio-Rad Laboratories, Richmond, CA, USA. Proteins separated on SDS-PAGE were electrophoretically blotted onto a nitrocellulose filter using a semidry system (Multiphor II Electrophoresis System; Pharmacia LKB Biotechnology, Uppsala, Sweden) as described by Harlow and Lane (34). After blotting, the filters were treated as described above (dot blot).

Blood collection and EPR measurements. Blood was collected from the orbital plexus of anesthetized mice with pipettes and tubes previously rinsed with heparin. Whole blood (700 μL) was pipetted into EPR quartz tubes (2.8 mm i.d. × 3.8 mm o.d.), and the samples were frozen in liquid nitrogen. The EPR spectra were obtained with a Bruker ER 200 D-SRC spectrometer using a finger-tip liquid nitrogen dewar. The spectrometer was operated at 9.77 Gz with a 100 kHz modulation frequency. The data were transferred to an IBM/AT computer where baseline subtraction and double integration over the total recorded magnetic field (600 G) were performed.

RESULTS

Murine cutaneous leishmaniasis can have distinct outcomes depending on the parasite species and mouse strain (28). As shown in Fig. 1, *L. amazonensis* amastigotes produced rapidly developing skin lesions in BALB/c mice as attested by the continuous increase in footpad thickness up to ulceration, which occurs after about 50 days for most animals. No sign of recovery was observed (Fig. 1) and after 100 days most mice had developed cutaneous metastatic lesions and visceralization of the parasites in the lymph nodes, spleen and liver (27).

At the time of ulceration, most cells present in the footpads are heavily parasitized macrophages (27), which could be producing nitric oxide and peroxynitrite (12–15). Hence, we examined the soluble protein fraction extracted from the footpads of infected animals for the presence of nitrotyrosine by dot immunoassay. Immunoreaction with polyclonal antibody against nitrotyrosine (18,19) was much higher in the protein fraction from infected than from naive mice (Fig. 2). Interestingly, homogenates from parasites drawn from the lesions (see Materials and Methods) were also reactive (Fig. 2), indicating that part of the immunoreactivity observed with proteins from the footpad was due to its infecting parasites. Normalization of the staining with protein concentration, however, indicates that host proteins are also nitrated during the course of the disease (Fig. 2). In all instances, antibody binding was completely inhibited by coincubation with 10 m*M* nitrotyrosine (18,19). Also, nonspecific antibody binding was excluded because no immunoreaction was observed when the anti-nitrotyrosine antibody was replaced

§*Abbreviations:* EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

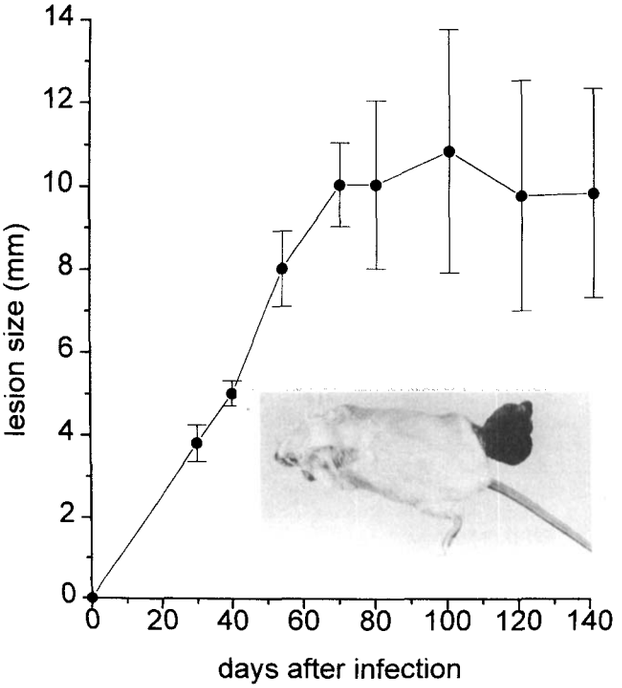

Figure 1. Evolution of murine cutaneous leishmaniasis in *L. amazonensis*-infected BALB/c mice. The animals were injected into the hind footpad with 2×10^6 amastigotes. Lesion size is expressed as the difference between the size of the infected and the contralateral uninfected footpad. The data represent the mean ± standard deviation of measurements of a group of 10 mice. The inset shows an animal after 62 days of infection.

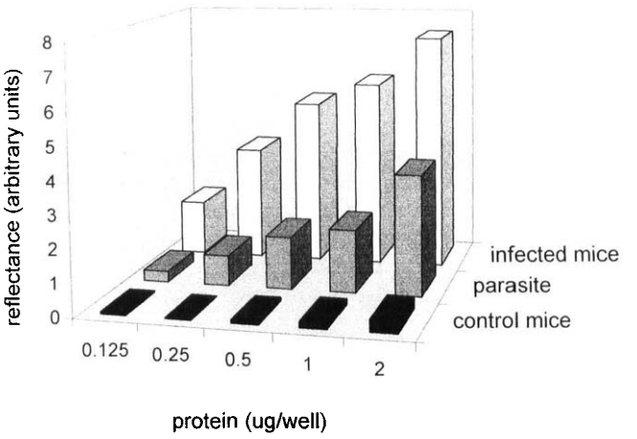

Figure 2. Relative intensity of the dot blot immunoreaction of protein homogenates from footpads of mice and from parasites with polyclonal anti-nitrotyrosine antibody. Reflectance of the spots was determined with a dual-wavelength flying-spot scanner.

with rabbit preimmune serum (not shown). Taken together, these results provide strong evidence for the formation of peroxynitrite in mouse lesions induced by *L. amazonensis.*

In an attempt to determine which proteins from both parasite and host could be preferentially nitrated, we performed western blot analyses of parasite and footpad homogenates (Fig. 3). An interesting observation was the fact that both homogenates from the infected mice and positive controls, obtained by treating the homogenates with authentic peroxynitrite, presented evidence of protein degradation by a relative increase in the low molecular weight protein bands (Fig. 3A). Protein fragmentation-mediated by peroxynitrite *in vitro* was recently reported (35). This degradation and the expected low specificity of protein tyrosine nitration by a reactive intermediate such as peroxynitrite are probably responsible for the smeared pattern obtained in the western blot (Fig. 3B). Immunoreaction, however, was observed in the case of proteins from infected mice and, to a lesser extent, from parasites (Fig. 3B) following the results obtained in the dot blots (Fig. 2). No immunoreactivity was observed in the case of proteins from naive mice (Fig. 3B) or from infected mice coincubated with nitrotyrosine (not shown). Consequently, although it was not possible to establish if any protein is preferentially nitrated in the lesion, these results confirm nitration of protein tyrosines during *L. amazonensis* infection and suggest that peroxynitrite-mediated protein fragmentation (35) may also occur *in vivo.*

Because formation of peroxynitrite in the cutaneous lesions of BALB/c mice infected with *L. amazonensis* (Figs. 2 and 3) should be preceded by nitric oxide generation (12–15), we decided to examine whole blood from the animals by EPR in an attempt to detect nitric oxide directly by its complexation with hemoglobin (36,37). As shown in Fig. 4, the EPR spectrum of the nitrosyl hemoglobin complex, with its characteristic three-line hyperfine structure (36,37) was easily detectable in infected animals (Fig. 4B–D) but not in naive mice (Fig. 4A). The levels of the nitrosyl hemoglobin

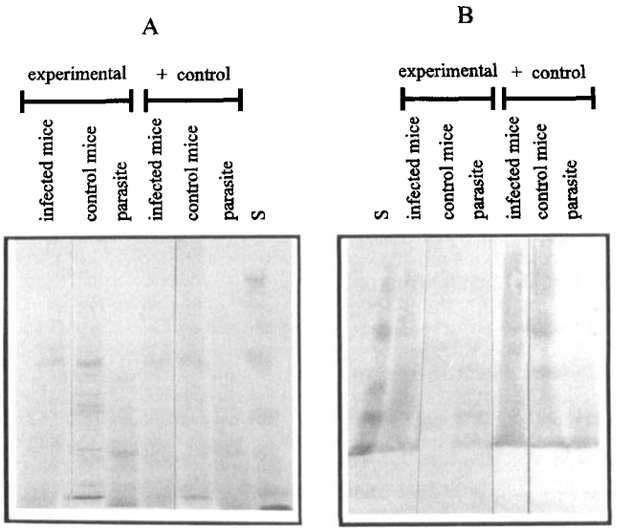

Figure 3. Analysis of protein homogenates from footpads of mice and parasites by PAGE (A) and western blot (B). Soluble proteins (25 μg/lane) were separated by PAGE (7–17%) (A) and electrophoretically blotted onto a nitrocellulose filter. After blotting, the filter was incubated with anti-nitrotyrosine antibody and developed by the peroxidase reaction (B) as described in the Materials and Methods. The labels in the figure lanes correspond to: S, kaleidoscope prestained protein standards; experimental, protein homogenates obtained from parasites or from footpads of infected and control mice, respectively; + control, the same homogenates displayed in experimental, which were preteated with peroxynitrite as described in the Material and Methods.

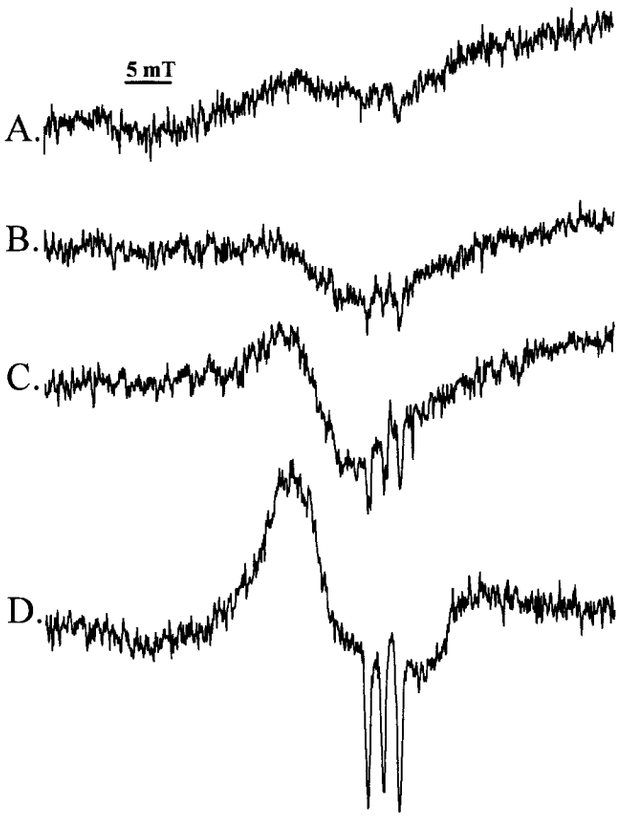

Figure 4. Representative EPR spectra of blood from BALB/c mice infected with *L. amazonensis*. A, Naive mouse; B, mouse after 35 days of infection; C, mouse after 64 days of infection and D, mouse after 148 days of infection. The spectra were run at 77 K. Instrumental conditions: microwave power, 10 mW; modulation amplitude, 0.5 mT; time constant 1 s; scan rate, 0.06 mT/s.

complex in the blood increased with aggravation of the disease (Figs. 1 and 4B–D). Indeed, the relative levels of nitrosyl hemoglobin obtained by integration of the EPR signals were 1:1.5:2.0 for BALB/c mice at 35, 64 and 148 days of infection, respectively; similar relative nitrosyl hemoglobin levels were obtained for a second group of infected animals. In the later stages of infection (148 days), nitrosyl hemoglobin could also be detected in the liver and spleen from infected mice (not shown).

DISCUSSION

The results reported here provide evidence for the formation of both, peroxynitrite (Figs. 2 and 3) and nitric oxide (Fig. 4) during murine leishmaniasis. This is the first direct demonstration of nitric oxide generation during *Leishmania* infection because we have detected the nitrosyl hemoglobin complex by EPR, whereas previous workers have used urinary nitrate excretion as a marker of nitric oxide formation (38).

Detection of the nitrosyl hemoglobin complex in the blood of BALB/c mice infected with *L. amazonensis* suggests a systemic production of nitric oxide. In agreement, nitrosyl hemoglobin complex levels became pronounced in the latter stages of the disease (Figs. 1 and 4), indicating that visceralization of the parasites elicits nitric oxide synthesis. Among the possible mediators of this synthesis are circulating lymphocytes that markedly increase nitric oxide production by endothelial cells *in vitro* (39).

Recent studies have compared the expression of inducible nitric oxide synthase during cutaneous infection elicited by *L. major* in the susceptible BALB/c mice with the resistant C57BL/6 mice (40). It was shown that inducible nitric oxide synthase appears earlier during infection and in significantly higher amounts in the cutaneous lesion and draining lymph nodes of the clinically resistant mice than in the nonhealing BALB/c mice. These results (40) are in agreement with previous studies that demonstrated that the susceptible BALB/c mice excreted less nitrate and, hence, formed less nitric oxide, than another *L. major* resistant mouse strain, C3H (38). All of these data provide support for the view that a pronounced expression of inducible nitric oxide synthase in resistant mice is an important mechanism for the elimination of *Leishmania in vivo* (38,40,41). It is important to note that our results do not challenge this view nor do they contradict previous studies that dealt with the initial stages of the infection (38,40). Indeed, urinary nitrate levels excreted by the resistant strain are much higher than those excreted by the susceptible strain up to about 20 days of infection (38). From then on, nitrate levels excreted by the resistant strain decrease, whereas those excreted by the susceptible strain increase, approaching a similar value for both strains at about 40 days of infection, the maximum follow-up time previously described (38). This is in good agreement with our measurements of the levels of nitrosyl hemoglobin complex in the blood (Fig. 4), supporting our conclusion that the late nitric oxide synthesis is triggered by visceralization of the parasites.

In addition to detecting the nitrosyl hemoglobin complex in the blood of infected mice we were able to demonstrate the presence of proteins containing nitrotyrosines in the cutaneous lesion and in the parasites drawn from it (Figs. 2 and 3). This is strong evidence for the formation of peroxynitrite, which in spite of being reactive (12–15) and leishmanicidal *in vitro* (23), was not able to eliminate the parasites, at least not at this point of the infection. On the contrary, peroxynitrite may be contributing to the host tissue damage including through its ability to induce protein fragmentation (Fig. 3A) (35). Undoubtedly, it will be necessary to follow both nitric oxide and peroxynitrite formation during the course of the infection in resistant and susceptible mice to discriminate between the leishmanicidal and host-damaging effects of peroxynitrite. At this point, however, it is clear that peroxynitrite can be formed during parasitic infections and that the oxidant and/or its precursor, nitric oxide, may play a dual role either combating (38,40,41) or aggravating the disease process.

Acknowledgements—O. Augusto acknowledges the privilege of performing her graduate studies under the supervision of Prof G. Cilento whose scientific achievements have been a continuous incentive for many pursuing high quality scientific work in the southern hemisphere. We thank Dr. J. S. Beckman (University of Alabama, Birmingham) for generously providing the anti-nitrotyrosine antibody. We also thank Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundo de Apoio ao Ensino e à Pesquisa da Unicamp and Financiadora de Estudos e Projetos for financial support.

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