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EFFECT OF HYPOXIA ON MACROPHAGE INFECTION BY *LEISHMANIA AMAZONENSIS*

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ABSTRACT: In the present study, we compared the effect of 5% oxygen tension (hypoxia) with a normal tension of 21% oxygen (normoxia) on macrophage infection by the protozoan parasite *Leishmania amazonensis*. Macrophages from different sources (human cell line U937, murine cell line J774, and murine peritoneal macrophages) exposed to hypoxia showed a reduction of the percentage of infected cells and the number of intracellular parasites per cell. Observations on the kinetics of infection indicated that hypoxia did not depress *L. amazonensis* phagocytosis but induced macrophages to reduce intracellular parasitism. Furthermore, hypoxia did not act synergistically with γ-interferon and bacterial lipopolysaccharides in macrophages to induce killing of parasites. Experiments also indicated no correlation between nitric oxide production and control of infection in macrophages under hypoxic condition. Thus, we have provided the first evidence that hypoxia, which occurs in various pathological conditions, can alter macrophage susceptibility to a parasitic infection.

Injury and infection, as well as various pathological conditions, can cause the formation of areas with low oxygen tension in tissues. Recently, there has been a renewed interest in the role played by the cellular response to hypoxia as a specific metabolic stimulus or signal (Yun et al., 1997; Lewis et al., 1999; Neubauer, 2001). Macrophages, cells involved in the clearance of microorganisms from infected tissues, antigen processing/presentation and, angiogenesis, are influenced by oxygen tension (reviewed by Lewis et al., 1999). Experimental hypoxia has been reported to influence cytokine secretion, expression of cell surface markers, migration, pinocytosis, and phagocytosis of inert particles (Leeper-Woodford and Mills, 1992; Metinko et al., 1992; Kalra et al., 1996; Leeper-Woodford and Detmer, 1999; Turner et al., 1999; Grimshaw and Balkwill, 2001; Reichner et al., 2001). However, to the best of our knowledge, no study has been conducted to establish the effect of hypoxia on macrophage infection by microorganisms.

Leishmaniasis is a vector-borne disease caused by the protozoan *Leishmania* spp., an obligate intramacrophage parasite. The severity of disease produced by *Leishmania amazonensis* ranges from localized, to diffuse cutaneous infection in humans (Grimaldi and Tesh, 1993; Hewaldt, 1999). The former is associated with a diffuse inflammatory process, followed by the development of granulomas, with infiltration of lymphocytes, epithelial cells, and macrophages infected with proliferating amastigotes and then secondary bacterial infection (El-On et al., 1992; Grimaldi and Tesh, 1993; Giorgio et al., 1998; Handman, 1999). In diffuse cutaneous lesions, where cure is not achieved, highly parasitized macrophages represent the main elements in the involved dermal areas (Grimaldi and Tesh, 1993). Several histological characteristics shared by wounds, tumors, delayed hypersensitivity type reaction, and leishmaniasis, such as an increased metabolic demand for leukocytes infiltrated into the inflammatory tissues, microcirculation impairment, and cell proliferation, result in the hypoxic environment observed in wounds, tumors, and skin delayed hypersensitivity reactions (Abbot et al., 1992; Lewis et al., 1999; Brown, 2002). Thus, it is likely that small regions of hypoxia also occur in leishmanial lesions and may play a role in the outcome of infection. The interaction between macrophages and *L. amazonensis* is a well-established in vitro model of intramacrophage parasitism (reviewed by Handman, 1999). The amastigote form of *Leishmania* spp. manages to survive and multiply within the phagolysosomes of nonactivated macrophages. The intracellular survival of the parasite is prevented when macrophages are stimulated with lymphokines, such as γ-interferon (IFN-γ) (reviewed by Solbach and Laskay, 2000). We, therefore, determined whether hypoxia altered macrophage susceptibility to *L. amazonensis* and their ability to kill intracellular parasites in response to macrophage-activating lymphokines.

MATERIALS AND METHODS

Reagents

Roswell Park Memorial Institute 1640 medium (RPMI), antibiotics, glutamine, *N*-2-hydroxythylpiperazine-*N′*-2-ethane-sulfonic acid (HEPES), phorbol myristate acetate (PMA), erythrosine B, trypan blue, Giemsa, recombinant mouse and human IFN-γ, and *Escherichia coli* lipopolysaccharides (LPS) were purchased from Sigma (St. Louis, Missouri). Fetal calf serum (FCS) was from Cultilab (Campinas, SP, Brazil). Cell culture plates, flasks, and glass coverslips were purchased from Nunc (Naperville, Illinois).

Parasite

Leishmania amazonensis (MHOM/BR/73/M2269) amastigotes were isolated from active skin lesions of BALB/c mice as described previously (Barbieri et al., 1993). Parasites were suspended in RPMI supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 100 mM HEPES, and 10% FCS and used immediately after isolation. Amastigote viability was determined by hemocytometer counts after staining with erythrosine B (Linares et al., 2001).

Cell cultures

The human promonocytic cell line U937 obtained from the American Type Culture Collection (Manassas, Virginia) was maintained in RPMI medium supplemented with antibiotics and 10% FCS at 37 C in a humidified incubator with 21% O_2, 5% CO_2, and balanced N_2. Two days before the experiments, cells were treated with 100 ng/ml PMA to induce differentiation and adherence (Martinez et al., 1988). The murine macrophage cell line J774 obtained from the American Type Culture Collection was maintained in RPMI medium supplemented with antibiotics and 10% FCS at 37 C in 21% O_2, 5% CO_2. Primary mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage, as described previously (Barbieri et al., 1993).

Hypoxia

Cells (5×10^5/ml U937, 10^5/ml J774 or 5×10^5/ml peritoneal macrophages) were added to 24-well culture plates containing 13-mm-diameter glass coverslips and incubated with RPMI medium supplemented with antibiotics and 10% FCS. The culture plates were then placed in a gas-tight modular chamber (Billups-Rothenberg, Del Mar, California). The chamber was gassed for 15 min at a flow rate of 2 L/min

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using certified gases containing O_2, CO_2, and N_2 (White Martins, Campinas, SP, Brazil) and placed in a 37 C temperature-controlled incubator. The percentage of O_2 was verified by measuring the outflow of gas at the end of the initial flushing period and then at 24-hr intervals using a Fyrite apparatus (Bacharach Inc., Pittsburgh, Pennsylvania). In all experiments, exposure of cells to 5% O_2, 5% CO_2, and balanced N_2 is referred to as hypoxia and exposure of cells to 21% O_2, 5% CO_2, and balanced N_2 is referred to as normoxia. The oxygen tension in the culture medium under hypoxia condition was 37 mm Hg and 150 mm Hg under normoxia condition (O_2 analyzer YSI/53, Yellow Springs Instruments Inc., Yellow Springs, Ohio). The pH of the medium was 7.4 and did not change significantly during the course of the experiments.

Cell viability tested by trypan blue exclusion, ability of adherence, cell growth (J774 cell line), and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) formazan production, a colorimetric dye-reduction assay (Mosmann, 1983), was determined under normoxic and hypoxic conditions. The viability of extracellular amastigotes exposed to normoxia and hypoxia conditions was determined by erythrosine B staining (Linares et al., 2001), MTT assay, and by the ability to transform into promastigote forms at 26 C (Lemesre et al., 1997).

Macrophage infection and activation

U937 and J774 macrophages were infected by adding to the cell cultures a suspension of *L. amazonensis* amastigotes in RPMI supplemented with antibiotics with a 2- and 10-fold excess of parasites for 24 hr, respectively. Peritoneal macrophages were infected with a 3-fold excess of parasites for 1 hr. After the infection period, cultures were washed to remove extracellular parasites and replaced with fresh medium (150 mm Hg oxygen tension) for the time of each experiment in normoxia and fresh medium (37 mm Hg oxygen tension) for the time of each experiment in hypoxia. Intracellular parasite destruction was assessed by morphological examination. Briefly, for the evaluation of the percentage of infected macrophages and the number of amastigotes per macrophage, cells on coverslips were stained with Giemsa. The intracellular amastigotes, which are exclusively localized in parasitophorous vacuoles, were examined microscopically at ×1,000 magnification (Giorgio and Barão, 1998). About 600 cells were counted per triplicate coverslip. Intracellular parasite killing was also quantified by a slight modification of a described technique (Mauel, 1984). Using this method, macrophage cultures were washed, lysed with 0.01% sodium dodecyl sulfate (SDS), and the amastigotes released. Viability was determined by erythrosine B staining. Amastigotes were also left to transform into promastigote forms, and after 3 days at 26 C, promastigote number was recorded by microscopic observation. In the experiments for macrophage activation, murine cells (J774 and peritoneal macrophages) were treated with 20 ng/ml mouse IFN-γ and 10 ng/ml LPS and U937 cells were treated with 500 UI/ml human IFN-γ and 100 ng/ml LPS 8 hr before *L. amazonensis* infection (Ho et al., 1992; Panaro et al., 1999; Linares et al., 2000).

Nitrite assay

Nitrite content in the supernatants of macrophage cultures was measured by the Greiss method (Green et al., 1984) using a colorimetric assay kit (Alexis Corporation, San Diego, California).

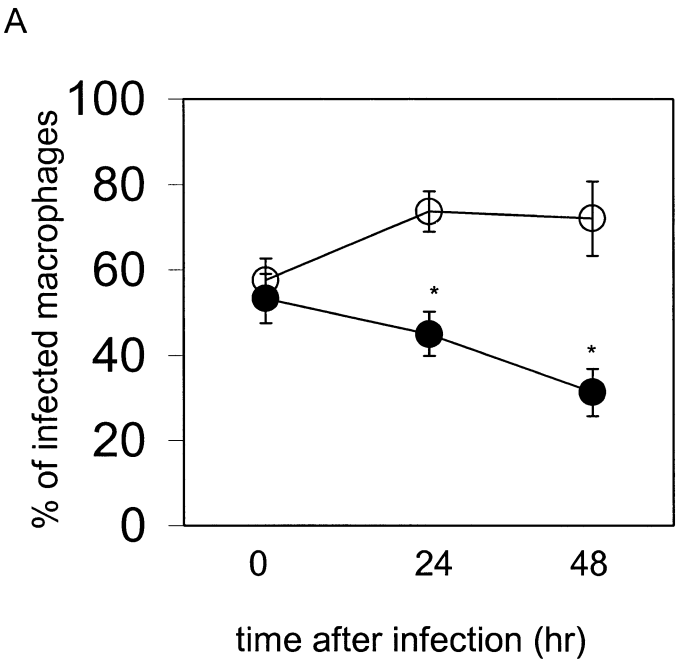
Results analyses

All experiments were repeated at least 3 times in triplicate wells. The results were expressed as mean ± SD. Data obtained with different conditions (hypoxia and normoxia) were analyzed statistically to determine whether any differences observed were significant ($P \leq 0.01$), using Student's *t*-test.

RESULTS

In a first set of experiments, we analyzed the effect of a 5% oxygen tension compared with a normal tension of 21% O_2 (normoxia) on *L. amazonensis* infection of primary macrophages obtained by peritoneal lavage from mice. Because this culture system is efficiently infected after 1 hr of parasite incubation, we set out to determine the effect of hypoxia on the kinetics of *L. amazonensis* in macrophages. For the assays, peritoneal macrophages were exposed to amastigotes for 1 hr under normoxia or hypoxia. The cultures were then washed and incubated an additional 0–48 hr under conditions of either normoxia or hypoxia. As shown in Figure 1, a similar percentage of macrophages contained intracellular parasites immediately after infection, irrespective of whether cells were cultured under normoxia or hypoxia. These results indicate that ingestion of parasites was identical for macrophages cultivated under normoxia or hypoxia. However, after 24 hr of culture under hypoxia, there was a significant reduction in the percentage of infected cells as well as in the number of amastigotes per cell compared with the normoxic condition (Fig. 1). A loss of macrophages from *L. amazonensis*–infected cultures (approximately 5%) attributed to detachment of heavily infected cells (Chang, 1980) was observed in normoxia as well as in hypoxia. The macrophages exposed to normoxia had large parasitophorous vacuoles containing amastigotes, which are characteristic of infection by this *Leishmania* species. In contrast, many macrophages in the population exposed to hypoxia had cleared their infection and had vacuoles containing no amastigotes (data not shown). Comparable results were obtained in similar experiments using a human monocytic cell line U937 and a murine cell line J774, i.e., low percentages of infected macrophages were observed in cultures exposed to hypoxia (23.6 ± 2.1% for U937 cell cultures, 21.3 ± 1.8% for J774 cell cultures), whereas cell cultures incubated with parasites under normoxia showed a significantly higher number of infected cells (49.1 ± 4.3% for U937 cell cultures, 43 ± 4.6% for J774 cell cultures) (Fig. 2). These results suggest that hypoxia led to macrophage destruction of intracellular *L. amazonensis* amastigotes. The intracellular parasite killing could also be assessed by quantification of the parasites recovered after lysis of the infected cells with 0.01% SDS. A significant 30% reduction in the number of viable amastigotes released from macrophages under hypoxia ($1.8 \pm 0.1 \times 10^5$/ml) was observed when compared with the number of viable amastigotes released from macrophages under normoxia ($2.6 \pm 0.08 \times 10^5$/ml). In addition, the ability of amastigotes released from macrophages under hypoxia to transform into promastigote forms after 3 days at 26 C ($2.5 \pm 0.8 \times 10^5$/ml promastigotes) was also significantly reduced when compared with amastigotes released from macrophages under normoxia ($5.6 \pm 0.2 \times 10^5$/ml promastigotes).

We addressed the question of whether the combination of hypoxia–normoxia conditions reduces the number of infected murine peritoneal macrophages. Cell cultures exposed to hypoxia at the time of infection (1 hr) followed by 48 hr of hypoxia showed a significant reduction in parasitized cells compared with cell cultures exposed to normoxia (36 ± 2.0% of infected macrophages vs. 74 ± 7.1% of infected macrophages). Likewise, macrophage cultures infected under conditions of normoxia and then incubated under hypoxia (24 hr) and normoxia (24 hr) exhibited a reduction in parasitized cells (32 ± 2.1% of infected macrophages). In contrast, cell cultures exposed to hypoxia only during the period of infection (1 hr) and then cultured under normoxia for the entire period of the experiment did not show a reduction in the percentage of parasitized cells (70 ± 5.8% of infected macrophages). Similar results were obtained with macrophage cultures preexposed to hypoxia for 24 hr and then infected and cultured under normoxia (68 ± 4.9%



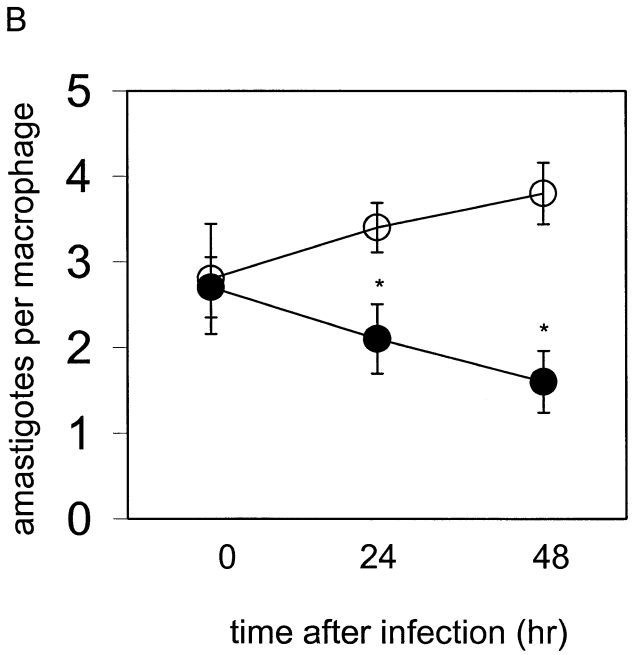


FIGURE 1. Effect of hypoxia on *Leishmania amazonensis* infection of murine peritoneal macrophages. Under normoxic (○) or hypoxic (●) conditions, peritoneal macrophages were infected with *L. amazonensis* amastigotes for 1 hr. Cell cultures were washed and immediately (0 hr) evaluated for percentage of infected macrophages and the number of amastigotes per macrophage or incubated under conditions of either normoxia or hypoxia for an additional 24 and 48 hr. The percentage of infected macrophages (**A**) and the number of amastigotes per macrophage (**B**). The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated in the figure. $*P \leq 0.01$.

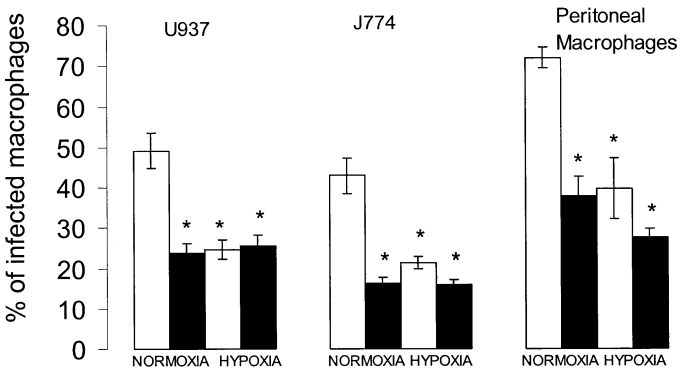


FIGURE 2. Effect of hypoxia on *Leishmania amazonensis* infection and IFN-γ plus LPS activation of macrophages. U937 and J774 cell lines and murine peritoneal macrophages were treated with IFN-γ plus LPS (■) or left untreated (□) for 8 hr. U937 and J774 cells were then infected with *L. amazonensis* amastigotes for 24 hr in normoxia or hypoxia. Peritoneal macrophages were infected with *L. amazonensis* amastigotes for 1 hr in normoxia or hypoxia and incubated under the same conditions (normoxia or hypoxia) for 48 hr. The percentage of infected macrophages was determined as described in Materials and Methods. The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in normoxia and cell cultures in hypoxia untreated or treated with IFN-γ plus LPS is indicated in the figure. $*P \leq 0.01$.

of infected macrophages). These data indicate that the relative resistance to *L. amazonensis* infection induced in macrophages by hypoxia is dependent on the combination of hypoxia–normoxia conditions and on the time that the cells remain in hypoxia (at least 24 hr after infection). In addition, the recovery of the ability of macrophages preexposed to hypoxia to support parasite replication after cultivation under normoxia indicates that these macrophages did not appear to be metabolically compromised.

It should be noted that viability was determined for every infected and uninfected cell culture (cell lines and primary macrophages) examined in these studies. Cell viability tested by trypan blue exclusion, cell growth, and the MTT assay was determined under normoxic and hypoxic conditions. Cell viability tested by trypan blue exclusion was approximately 90% at all time points after exposure to normoxia and hypoxia. The cell growth was similar in uninfected J774 cell line cultured under normoxia and hypoxia (data not shown) as also were the results of the MTT assay for primary macrophages (MTT formazan production 48 hr after hypoxia was 93 ± 8% of MTT formazan production 48 hr after normoxia). In addition, to determine whether the reduction in *L. amazonensis* infection in macrophages exposed to hypoxia (Figs. 1, 2) could be attributed to a loss of amastigote viability during the period of incubation under hypoxia, we measured their erythrosine B staining, MTT cleavage, and ability to transform into promastigote forms. The viability and ability of transformation were not reduced in extracellular amastigotes preincubated for 24 hr under hypoxia (data not shown), and their MTT formazan production was also unchanged (111 ± 13% of MTT formazan production of extracellular amastigotes preincubated for 24 hr under normoxia).

Leishmania spp. infection experiments have shown that IFN-γ activates macrophage destruction of intracellular parasites in the presence of LPS (Corradin and Mauel, 1991; Panaro et al., 1999). We, therefore, compared the effect of hypoxia and IFN-

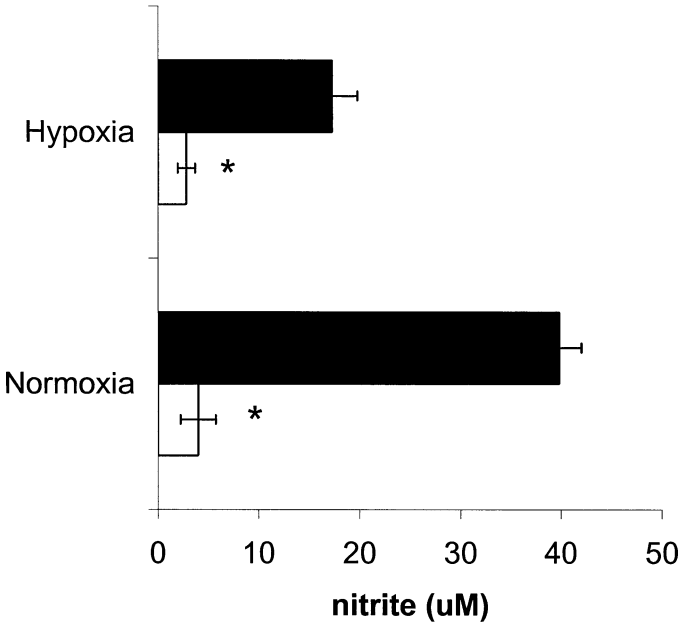


FIGURE 3. Effect of hypoxia on nitrite production by macrophages infected with *Leishmania amazonensis* and activated with IFN-γ plus LPS. Murine macrophages were treated with IFN-γ plus LPS (■) or untreated (□) during 8 hr in normoxia. Cells were infected with *L. amazonensis* amastigotes and incubated for 48 hr under normoxia or hypoxia. Nitrite concentrations were determined in the culture supernatants by the Griess reaction as described in Materials and Methods. The results represent the mean ± SD of 3 experiments. The significance of the difference between cell cultures in hypoxia and normoxia is indicated in the figure. $*P \leq 0.01$.

γ plus LPS on macrophage infection by *L. amazonensis*. As demonstrated in Figure 2, U937 and J774 cell lines and murine peritoneal macrophages treated with the activators before infection and incubated under normoxia showed decreased numbers of parasitized cells. These data agree with those previously reported for these cell systems (Corradin and Mauel, 1991; Panaro et al., 1999). When macrophage cultures were previously activated with IFN-γ plus LPS and then infected with *L. amazonensis* under hypoxic conditions, cells were able to kill intracellular parasites with an efficiency comparable to that shown by IFN-γ plus LPS-activated cells or cells exposed to hypoxic conditions only (Fig. 2). These latter results indicate that hypoxia worked as well as IFN-γ plus LPS and did not act synergistically with these activators to induce killing of *L. amazonensis* by macrophages.

Because in vitro and in vivo studies suggest that nitric oxide is implicated as 1 of the major effector molecules for lymphokine-activated macrophage killing of *Leishmania* spp. (Green et al., 1990; Panaro et al., 1999; Linares et al., 2001), we next examined the effect of hypoxia on the nitric oxide production (measured as nitrite) of infected cells. As shown in Figure 3, macrophages infected with *L. amazonensis* released low levels of nitrite when incubated under normoxia or hypoxia (<4 μM). The combination of IFN-γ plus LPS stimulated macrophages to produce nitrite after *L. amazonensis* infection and under normoxic conditions (39.8 ± 2.2 μM). However, *L. amazonensis*–infected cells exposed to hypoxia after IFN-γ plus LPS activation produced less nitrite than those exposed to normoxia (17.3 ± 2.5 μM vs. 39.8 ± 2.2 μM) (Fig. 3). These results suggest no apparent correlation between nitrite concentration and the reduction of intracellular parasitism observed in macrophages maintained under hypoxia (Figs. 1, 2) and also indicate an inhibitory effect of hypoxia on nitric oxide production by macrophages activated with IFN-γ plus LPS and infected with *L. amazonensis* (Fig. 3).

DISCUSSION

The present experiments were conducted to investigate the effect of hypoxia (5% O_2) on macrophage susceptibility to *L. amazonensis*. Under the conditions used, we provide evidence that macrophages from different sources (cell lines and primary cells) exposed to hypoxia and infected with *L. amazonensis* showed a reduction in the percentage of infected cells and in the number of intracellular parasites per macrophage (Figs. 1, 2). The kinetics of infection in murine peritoneal macrophages indicated that hypoxia did not depress *L. amazonensis* phagocytosis but induced macrophage resistance to the parasitic infection (Fig. 1).

The absence of differences in viability and adherence between infected macrophages cultured under hypoxia and under normoxia (see results) indicates that these factors did not play a significant role in the effect of hypoxia on the cell response to parasitic infection in our experiments. It should be noted that recovery of the ability to support parasite replication after exposure to normoxia indicated that macrophages preexposed to hypoxia did not appear to be metabolically compromised (see Results). These latter results are consistent with those reported by several authors who showed that hypoxia (1.7–6% O_2) did not affect significantly macrophage viability, adherence, or cell growth (Leeper-Woodford and Mills, 1992; Turner et al., 1999; McCormick et al., 2000). Although we cannot exclude that hypoxia alters amastigote metabolism during the periods of incubation with macrophages in hypoxia, our results indicate that viability and capacity to transform into promastigote forms are maintained in extracellular amastigotes preexposed to hypoxia (see Results).

No reports of the effect of hypoxia on macrophage infection by microorganisms are available for comparison with our results. However, a number of studies have reported inhibitory effects of hypoxia on the phagocytic activity of macrophages. Alveolar macrophages showed reduced uptake and retention of fixed red blood cells when exposed to hypoxia (1.7% O_2) (Leeper-Woodford and Mills, 1992). Rat macrophages obtained from wounds and from the peritoneum, and cultured under hypoxia, showed reduced phagocytosis of zymozan particles and latex beads (Reichner et al., 2001). In contrast, Turner et al., 1999 did not observe the influence of hypoxia on human monocyte cell line phagocytosis of opsonized red blood cells, and Matsumoto et al., 2000 showed that there was no difference in binding and uptake of low-density lipoprotein (LDL) in human monocyte-derived macrophages. The reasons for the different results might be attributed to the array of different macrophage types tested, the extent and duration of hypoxia applied, and the nature of the particles used in the various studies. Nevertheless, it appears that hypoxia did not affect *L. amazonensis* entry into macrophages (Fig. 1). We propose that in this cell system hypoxia did not induce marked changes in ligands, re-

ceptors, or cytoskeletal proteins of either macrophage or parasite that could have an effect on the uptake of *L. amazonensis* by macrophages. On the other hand, hypoxia induced macrophage cultures to inhibit the growth of, or kill (or both), intracellular parasites (Figs. 1, 2). These results are comparable to those obtained in normoxic cultures treated with IFN-γ plus bacterial LPS, a well-defined activator system of leishmanicidal mechanisms in macrophages (Fig. 2) (Corradin and Mauel, 1991; Panaro et al., 1999; Solbach and Laskay, 2000). Interestingly, the macrophages activated with IFN-γ plus LPS and infected with *L. amazonensis* under normoxic or hypoxic conditions were able to reduce intracellular parasitism with the same efficiency, indicating that hypoxia works as well as IFN-γ plus LPS and did not act synergistically with these activators to induce killing of *L. amazonensis* (Fig. 2).

Several studies have shown that despite inducible nitric oxide synthase gene activation, nitric oxide synthesis is decreased in proportion to oxygen tension (Albina et al., 1995; Melilo et al., 1996; McCormick et al., 2000; Otto and Baumgardner, 2001). However, the fact that nitric oxide is an important active component engaged in the killing of *L. amazonensis* in lymphokine-activated macrophages (Green et al., 1990; Panaro et al., 1999; Linares et al., 2001) shows that the measurement of nitrite production is valuable to investigate the leishmanicidal mechanism of macrophages under hypoxia. Our results indicate that hypoxia reduces the synthesis of nitrite in IFN-γ plus LPS-activated cells infected with *L. amazonensis* by at least 50% (Fig. 3). These results are similar to those of McCormick and colleagues who showed a 60–50% decrease in nitrite production in IFN-γ plus LPS-activated (uninfected) peritoneal macrophages (McCormick et al., 2000). Perhaps more importantly, our data indicate no correlation between nitrite production (<4 μM) and the reduction in infection in macrophages under hypoxia. Although we cannot exclude the participation of the other reactive species generated by oxidative reactions in the anti–*L. amazonensis* activity of macrophages under hypoxia, this seems unlikely because limited O_2 availability as a substrate is observed during hypoxia (McCormick et al., 2000; Otto and Baumgardner, 2001). The data presented here do not explain the precise mechanism by which macrophages are able to control *L. amazonensis* infection in hypoxia and further investigations are needed to clarify this point.

To summarize, we have shown that hypoxia induces macrophage resistance to *L. amazonensis* but does not impair ingestion of the parasite. Furthermore, hypoxia worked as well as IFN-γ plus LPS and did not act synergistically with these activators to induce killing of parasites. Our data suggest no correlation between nitric oxide production and control of infection in macrophages under hypoxic conditions. Whether the effects of hypoxia on macrophages observed in vitro play a role in tissue damage occurring during the course of leishmaniasis is a question to be addressed.

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