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Infection, Genetics and Evolution 6 (2006) 187–197
Infection, Genetics and Evolution
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Leishmania infantum amastigotes resistant to nitric oxide cytotoxicity: Impact on in vitro parasite developmental cycle and metabolic enzyme activities

Philippe Holzmuller [a,*], Mallorie Hide [b], Denis Sereno [a], Jean-Loup Lemesre [a]

[a] *UR 008 Pathogénie des Trypanosomatidés, IRD (Institut de Recherche pour le Développement), B.P. 64501, 911 avenue Agropolis, 34394 Montpellier cedex 5, France*
[b] *UMR CNRS/IRD 2724, Génétique et Evolution des Maladies Infectieuses, IRD (Institut de Recherche pour le Développement), B.P. 64501, 911 avenue Agropolis, 34394 Montpellier cedex 5, France*

Received 9 February 2005; received in revised form 22 March 2005; accepted 22 March 2005
Available online 17 May 2005

Abstract

Nitric oxide (NO) has been demonstrated to be the principal effector molecule mediating intracellular killing of *Leishmania*. The free radical characteristic of NO prevented direct induction of resistance in *Leishmania* wild-type parasites. Starting from the previous observation that antimony-resistant amastigotes of *Leishmania infantum* were not affected by NO-induced apoptotic death, we used a continuous NO pressure protocol and succeeded in inducing NO resistance in amastigote forms of *L. infantum*. Two clones resistant to 50 μM (LiNOR50) and 100 μM (LiNOR100) of the NO donor DETA/NONOate, derived from parental clone weakly resistant to trivalent antimony (LiSbIIIR4), were selected and analysed. Both clones were also resistant to other NO donors, particularly SNAP. In the absence of potassium antimonyl tartrate, all clones (LiSbIIIR4, LiNOR50 and LiNOR100) lost their antimony resistance almost totally. Interestingly, the parasitic developmental life cycle of NO-resistant mutants was dramatically disturbed. NO-resistant amastigotes differentiated more rapidly into promastigotes than the wild-type ones. Nevertheless, NO-resistant amastigotes produce a maximal number of parasites 1.5–2 times lower than the wild-type whereas, after differentiation, NO-resistant promastigotes produced more cells than the wild-type. We showed that this last phenomenon could be a consequence of the overexpression of parasitic enzymes involved in both glycolysis and respiration processes. NO-resistant amastigotes overexpressed three enzymes: *cis*-aconitase, glyceraldehyde-3-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The two first enzymes are NO molecular targets which could be directly involved in NO resistance and the third one could interfere in modifying *Leishmania* metabolism.

Keywords: *Leishmania*; Nitric oxide; Chemoresistance; Developmental cycle; Apoptosis; Enzyme overexpression; *cis*-Aconitase; G3PDH; 6PGDH

1. Introduction

Leishmania is a trypanosomatid, a flagellated parasitic protozoon that causes a variety of diseases in mammals including humans in tropics and subtropics area. The parasite alternates between sandfly and mammalian hosts and has two developmental forms. The promastigote is a motile, flagellated form that multiplies in the sandfly gut; the metacyclic promastigote is a nondividing infective form that resides in the sandfly mouthparts. The amastigote is a nonmotile form that lives and replicates in the phagolysosomal compartment of mammalian macrophages (Pearson et al., 1983).

In experimental murine leishmaniasis, a close correlation has been observed between the outcome of infection and the type of T cell response involved. Susceptibility to infection was associated with activation of Th2 cells secreting interleukine (IL)-4, IL-5, IL-6 and IL-10. By contrast, when T cell response is predominated by Th1 cells producing IL-12, interferon-γ (IFN-γ) and lymphotoxin, the animals succeeded in eliminating the parasite and controlling the infection (Scott, 1991). Such dichotomic immunity against *Leishmania* begins to be demonstrated in human and canine

* Corresponding author. Tel.: +33 4 67 41 61 63; fax: +33 4 67 41 63 30.
E-mail address: holzmull@mpl.ird.fr (P. Holzmuller).

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doi:10.1016/j.meegid.2005.03.003

models also (Vouldoukis et al., 1995, 1996; Mossalayi et al., 1999; Panaro et al., 1999, 2001; Pinelli et al., 1994, 2000). Cytokines-activated macrophages can generate large amounts of nitric oxide (NO), which has many biological functions including defense against intracellular pathogens and particularly *Leishmania* (reviewed in Nathan and Shiloh, 2000; Brunet, 2001; Colasanti et al., 2002). Recently, we demonstrated that NO killed *Leishmania* parasites by inducing amastigotes apoptosis (Holzmuller et al., 2002). Moreover, some molecular targets have been previously shown to be inhibited by NO, such as *cis*-aconitase (Lemesre et al., 1997), glyceraldehyde-3-phosphate dehydrogenase (Mauel and Ransijn, 1997) or cysteine proteinases (Salvati et al., 2001), and may be involved in initiating programmed cell death.

Several pathways are involved in drug resistance in *Leishmania*. Resistant strains can be obtained in vitro by increasing the drug pressure stepwise. These studies have recently shown that parasites could modify their membrane properties to face the toxicity of the drug (Chiquero et al., 1998; Kundig et al., 1999), but more often an amplification of the gene encoding the target enzyme of the drug was demonstrated (reviewed in Segovia, 1994; Haimeur et al., 1999; Yan et al., 2001; Guimond et al., 2003; Anacleto et al., 2003). By contrast, little is known about resistance to natural leishmanicidal molecules such as NO. In *Leishmania*, mechanisms described for resistance to nitric oxide action mainly involved the modulation of inducible nitric oxide synthase (NOS II) mRNA expression or enzymatic activity by parasite molecules (Stenger et al., 1996; Piani et al., 1999; Linares et al., 2000; Balestieri et al., 2002). NO could also induce expression of stress proteins (heat shock proteins 83, 70 and 65) that may confer to the parasite a protection against its toxic effects (Adhuna et al., 2000), but nothing is known about the ability of *Leishmania* parasites to directly resist NO injuries. In a rat model, a study demonstrated that the protecting mechanism from NO neurotoxicity in dopaminergic neurons is based on inhibition of conversion of NO to peroxynitrite anion (Sawada et al., 1996). More recently, resistance to NO was ascribed to the maintenance of ATP content in NO-treated glucose-fed human epithelial cells. This resistance was due to their ability to derive their energy from anaerobic glycolysis (Le Goffe et al., 2002).

In this study, we describe an original phenotype of *Leishmania infantum* amastigotes which is resistant to NO-mediated apoptotic cell death. Impact on kinetics properties and metabolic enzyme activities are analysed and discussed.

2. Materials and methods

2.1. Reagents

Foetal calf serum (FCS) was obtained from Dutscher S.A. RPMI 1640 medium (Lot. 0MB0174) was purchased from Bio Whittaker Europe and L-glutamine (Lot. 3414) from Bio Media. Penicillin/Streptomycin (10,000 IU/ml–10,000 UG/ml, Lot. 3037222) and Phosphate Buffer Saline (Ca^{2+}/Mg^{2+} free) were obtained from Life Technologies. Bacterial lipopolysaccharid (LPS), interferon-γ (Mouse recombinant), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, Lot. 66H5033) and potassium antimonyl tartrate trihydrate were purchased from Sigma. NO donors *S*-nitroso-*N*-acetylpenicillamin (SNAP) and (*Z*)-1-[2-(2-Aminoethyl)-*N*-(2-ammonioethyl)amino]-diazen-1-ium-1,2-diolate (DETA/NONOate) were purchased from Alexis Biochemicals. All other cellular and molecular biology grade chemicals were obtained from Sigma.

2.2. Animals

Female Balb/C and C57/Black6 mice (Iffa Credo, Saint-Germain-sur-l'Arbresle, France) were housed under conventional conditions and given water and chow ad libitum. Experimentation on animals was conformed to institutional guidelines.

2.3. Parasites and in vitro cultures

A cloned line of *L. infantum* (MHOM/MA/67/ITMAP-263) was used in all experiments. *L. infantum* axenically-grown amastigote forms were maintained at 37 ± 1 °C by weekly subpassages in MAA/20 medium (Lemesre et al., 1994, 1997). From a starting inoculum of 5×10^5 amastigote forms/ml, cell density of about 2-3 $\times 10^8$ parasites/ml was obtained on day 7. Axenically-grown amastigote forms appeared homogeneous, round to ovoid, without apparent flagellum and non motile. Axenically-grown amastigotes from various *Leishmania* species clearly resembled intracellular amastigotes with regard to their ultrastructural, biological, biochemical and immunological properties (Lemesre et al., 1994, 1997; reviewed in Gupta et al., 2001).

Promastigote cultures were derived from axenically-grown amastigote stages by subpassage at 25 ± 1 °C in medium RPMI 1640 (Gibco BRL) buffered with 25 mM HEPES and 2 mM $NaHCO_3$, pH 7.2, supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). Initial parasite concentration was 1×10^6 living amastigotes per ml of medium for the differentiation experiments. The in vitro parasitic developmental life cycle was achieved by subpassages of 1×10^6 resulting promastigotes in MAA/20.

Cell concentrations were determined daily by Facscan™ flow cytometry after propidium iodide staining and adequate dilution (1/100) in Facsflow solution (Becton Dickinson, Ivry, France). The transformations of extracellular amastigote forms into promastigote ones and of promastigotes into amastigotes were estimated microscopically after methanol fixation and Giemsa staining.

2.4. Selection of NO-resistant amastigotes of L. infanum

We have previously demonstrated the existence of a cross-resistance between NO and potassium antimony tartrate in *L. infantum* amastigotes (Holzmuller et al., in press). Cloned amastigotes, weakly resistant to potassium antimonyl tartrate (4 μg/ml) (Sereno et al., 2000) were adapted to survive in culture medium containing about 0.1 μM DETA/NONOate. After 10 subcultures in the medium containing the same NO concentration, cells were cultured in medium containing increasing amounts of DETA/NONOate. These cells were subjected to stepwise increasing NO donor pressure until cell lines resistant to 50 and 100 μM DETA/NONOate were established respectively. Cells were then cloned and susceptibility of the clones to DETA/NONOate was determined using the MTT-based microassay (used as described before by Sereno et al., 1997). The two selected clones, designated as LiNO50 and LiNOR100, were then cultured in MAA/20 in the presence of 50 μM and 100 μM DETA/NONOate, respectively.

2.5. Viability test

IC_{50} values were estimated by MTT test. Tetrazolium bromide has the property to be reduced by parasisitic dehydrogenases into a dark-blue insoluble formazan product, the amount of which depends on the number of viable amastigotes. Briefly, amastigotes from late-log phase of growth were seeded in 96-well microplates at 2×10^5 parasites/well in a volume of 90 μl in adequate culture conditions. Drugs were added at 1/10 of the final concentration, and the range depending on the NO donor used (10–500 μM for SNAP and 10–200 μM for DETA/NONOate). After 72 h incubation, 10 μl of MTT (10 mg/ml) were added to each well and plates were further incubated for 4 h. Hundred microliters of 50% isopropanol–10% SDS were added to stop the enzymatic reaction. The plates were incubated for an additional 30 min step under agitation at room temperature. OD values were read at 570 nm with a titter-tech 96-well scanner (Labsystems Multiscan EX). Activity of the NO donors alone in culture medium was determined and no substantial interaction was found. Three independent experiments were performed in triplicate to determine amastigotes sensitivity to each NO donor. Results were analysed using the mathematical model described by Hills (1986).

2.6. In vitro macrophages infections

Peritoneal macrophages of Balb/C (leishmaniasis susceptible model) or C57/Black6 (leishmaniasis resistant model) mice were washed with pre-warmed RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and cultured overnight in 16 wells Lab-Tek® tissue culture slides (Nalge Nunc International). Non-adherent cells were removed by washing twice with pre-warmed RPMI medium and then macrophages were infected with stationary-phase extracellular amastigotes of each clone (WT, LiNOR50, LiNOR100) at a parasites:macrophage ratio of 3:1 for 2 h at 37 °C with 5% CO_2. Non-internalised parasites were removed by gently washing in pre-warmed RPMI medium. Infected macrophages were then cultured in the presence or absence of lipopolysaccharide (LPS, 10 ng/ml) and interferon-γ (IFN-γ, 100 U/ml), to activate cells for NO production.

After 48 h, culture supernatants were collected for nitrite production measurement. Macrophages were then washed with pre-warmed RPMI, fixed with methanol and stained with Giemsa for parasite counts or processed for the TUNEL technique (see below). The parasitic index (PI) was calculated, PI = 100 − [(mean number of amastigotes per macrophage × percentage of infected macrophages in treated wells)/(mean number of amastigotes per macrophage × percentage of infected macrophages in untreated wells)] × 100.

2.7. Measurement of nitrite production

For the leishmanicidal assay, NO_2^- accumulation in the medium after 48 h was used as an indicator of NO production and was assayed by the Griess reaction (Roy and Wilkerson, 1984). Briefly, in a 96-well plate, 60 μl Griess reagent A (sulfanilamide 1% in HCl 1.2N) and 60 μl Griess reagent B (*N*-(1-naphtyl)-ethyl-enediamine 0.3%) were added to 100 μl of each supernatant in triplicate wells. Plates were read at 540 nm in an ELISA plate reader (Labsystems Multiskan EX). $NaNO_2$ in RPMI was used to construct a standard curve for each plate reading.

2.8. In situ terminal deoxynucleotidyl transferase-mediated dUTP Nick-End labeling (TUNEL technique)

The in situ TUNEL was performed as previously described for *L. amazonensis* submitted to NO donors or *L. infantum* treated with antimonials (Sereno et al., 2001; Holzmuller et al., 2002). Briefly, slides with infected macrophages were fixed for 20 min with PBS containing 4% paraformaldehyde, washed twice with PBS 0.01 M pH 7.2 and stored at −20 °C until use. DNA fragmentation was analysed in situ using a colorimetric apoptosis detection system (Promega, Madison, USA), according to the manufacturer's instructions. After the TUNEL protocol, preparations were analysed using a microscope at 1000× magnification. Apoptotic nuclei and kinetoplasts appeared dark brown. Other nuclei were not coloured.

2.9. Multilocus enzyme electrophoresis technique (MLEE) analysis

MLEE is a method usually used for genetic studies (Richardson et al., 1986) but can be useful for quantitative

comparisons of the expression of metabolic enzymes as previously described (Allen et al., 1998; Terao et al., 2000). Experiments were performed on cellulose acetate plates (Helena Laboratories) according to the method described by Ben Abderrazak after slight modifications (Ben Abderrazak et al., 1993). For each clone (WT, LiNOR50, LiNOR100), amastigotes cultures were harvested at day 3 (one pellet) and day 6 (three pellets derived from three independent experiments) and stored immediately at −80 °C. Amastigote pellets were lysed in an equal volume of enzyme stabilizer (EDTA, dithiotreitol, ϵ-aminocaproic acid, at 2 mM) on an ice bed for 15 min. The lysates were centrifuged at 13,000 × *g* for 10 min at 4 °C and then the water-soluble fraction was removed and stored at −80 °C until use. The two NO target enzymes (Lemesre et al., 1997; Mauel and Ransijn, 1997) as well as seven control systems were studied; they were as follows: *cis*-aconitase hydratase (ACON: E.C.4.2.1.3), glyceraldehyde-3-phosphate dehydrogenase (G3PD: E.C.1.2.1.12), 6-phosphogluconate dehydrogenase (6PGDH: EC1.1.1.44); superoxide dismutase (SOD: E.C.1.15.1.1); glucose phosphate isomerase (GPI: E.C.5.3.1.9); glutamate oxaloacetate transaminase (GOT: E.C.2.6.1.1); isocitrate dehydrogenase (NADP+) (IDH: E.C.1.1.1.42); malic enzyme (ME: E.C.1.1.1.40); mannose-6-phosphate isomerase (MPI: E.C.5.3.1.8); nucleoside hydrolase (inosine) (NHI: E.C.2.4.2.*).

3. Results

3.1. Obtaining of NO-resistant amastigote forms of *L. infantum*

Table 1 shows the levels of resistance to NO donors (resistance index RI = 1.8, RI represents the IC_{50} value of NO-resistant clone/IC_{50} of wild-type clone for the molecule considered) of amastigotes weakly resistant to potassium antimonyl tartrate (LiSbIIIR4). Starting from this cloned amastigotes and using a continuous NO donor pressure protocol (DETA/NONOate), we selected NO-resistant amastigotes with increasing RI ranging from 3.2 for LiNOR50 to 5 for LiNOR100 (Table 1). As expected, both NO-resistant clones cross-resisted other NO donors, such as SNAP, with resistance indexes of 3.3 and 5.4 for LiNOR50 and LiNOR100, respectively. RI to potassium antimonyl tartrate was reduced by 73.9% for LiNOR50 and by 68.9% for LiNOR100 as compared to the LiSbIIIR4 clone used to initiate the NO pressure (Table 1).

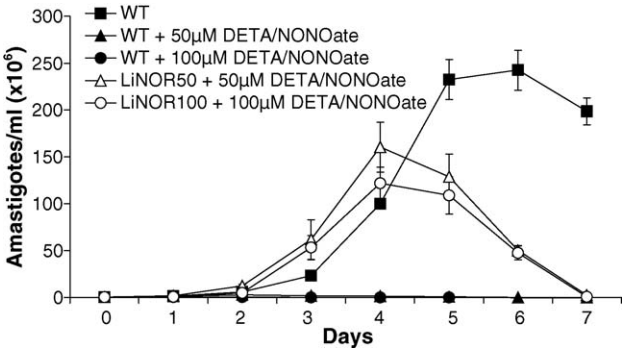

Fig. 1. *Leishmania infantum* NO-resistant amastigotes growth. Starting from an inoculum of 5×10^5 parasites per ml, proliferation experiments are performed in the presence or absence of 50 μM or 100 μM DETA/NONOate (concentrations used to subculture NO-resistant clones). Results are expressed as the mean and standard deviation of three independent experiments performed in triplicate.

3.2. Growth of NO-resistant amastigotes of *L. infantum*

As shown in Fig. 1, both LiNOR50 and LiNOR100 amastigotes clones of *L. infantum* were less fit to grow than the wild-type clone. On the one hand, resistant amastigotes entered earlier in stationary phase of growth, as early as the resistance index was high (Fig. 1) and on the other hand, they produce a maximal number of parasites 1.5–2 times lower than the wild-type. Nevertheless, the wild-type clone could not readily grow at the NO donor concentrations corresponding to LiNOR50 and LiNOR100 clones (Fig. 1). By contrast, both LiNOR50 and LiNOR100 clones were able to grow in the presence of the NO donor exhibiting growth curves similar to those obtained in a drug-free medium (data not shown). Moreover, kinetics of NO-resistant clones are reproducible for at least 10 subpassages in a drug-free medium, whereas LiNOR10 and LiNOR25 corresponding to clones of *L. infantum* resistant to 10 and 25 μM DETA/NONOate respectively, used in the stepwise increasing NO pressure, decreased their RI values when cultured for 10 weeks in the

Table 1
Effects of antimonials and NO donors on wild-type, trivalent antimony-resistant and NO-resistant *Leishmania infantum* amastigotes clones

	IC_{50} (μM)[a]				Relative drug resistance (RI)		
	WT	LiSbIIIR4	LiNOR50	LiNOR100	LiSbIIIR4	LiNOR50	LiNOR100
SNAP	72 ± 0.8	132.4 ± 8.1	233.3 ± 17.6	388.8 ± 26.2	1.8	3.2	5.4
DETA/NONOate	37.7 ± 4.25	67.6 ± 1.9	126 ± 1.3	188 ± 4	1.8	3.3	5
Potassium antimonyl tartrate[b]	4.7 ± 2.4	56.04 ± 17.7	14.8 ± 2.7	17.3 ± 3.6	11.9	3.1	3.7

The effective concentrations of antimony-containing drug and NO donors that inhibit the growth of wild-type (WT), trivalent antimony-resistant (LiSbIIIR4) and NO-resistant (LiNOR50 and LiNOR100) amastigotes by 50% (IC_{50}) were determined by the MTT-based microassay (Sereno and Lemesre, 1997). RI: resistance index (IC_{50} of resistant parasites/IC_{50} of wild-type parasites). SNAP: *S*-nitroso-*N*-acetylpenicillamin. DETA/NONOate: (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate.

[a] Values are expressed as mean ± standard deviation ($n = 3$).

[b] IC_{50} values of potassium antimonyl tartrate are expressed in μg/ml.

absence of DETA/NONOate pressure (data not shown). We thus considered that the NO resistance induced for LiNOR50 and LiNOR100 clones was stable in the time course of our experiments.

3.3. In vitro parasite life cycle of NO-resistant amastigotes

Fig. 2 indicates that both NO-resistant clones were able to complete the two sequences of differentiation (i.e. promastigote-amastigote-promastigote) representing the in vitro life cycle of *Leishmania* parasites. Interestingly, NO-resistant amastigotes differentiated more rapidly into promastigotes than the wild-type one. After 24 h in promastigote culture medium, 33 and 46% of LiNOR50 and LiNOR100, respectively, were transformed, whereas wild-type amastigotes show only 5% differentiation (Fig. 2A). Moreover, 100% of LiNOR100 and 99% LiNOR50 amastigotes were differentiated into promastigotes within 3 days, while full differentiation process took 4 days for wild-type amastigotes (Fig. 2A). The same kind of speeding up was observed for the promastigote-amastigote differentiation sequence (Fig. 2C). In day 3, wild-type, LiNOR50 and LiNOR100 promastigotes showed 54, 69 and 96% differentiation, respectively. They were almost all fully transformed after 4 days (Fig. 2B).

As a consequence of their earlier differentiation, promastigotes resulting from LiNOR100 amastigotes produced more cells than those resulting from LiNOR50 amastigotes, which produced more cells than the wild-type ones (Fig. 2B). After subpassages, kinetics of promastigotes derived from NO-resistant amastigotes maintained a faster growth curve compared to wild-type promastigotes as observed after amastigote-promastigote differentiation (data not show). By contrast, NO-resistant amastigotes resulting from the promastigote-amastigote differentiation exhibited attenuated growth curves compared to the wild-type one (Fig. 2D). Interestingly, both LiNOR50 and LiNOR100 showed an early entry in stationary phase of growth, as previously observed in the proliferation experiments (Figs. 1 and 2D).

Interestingly, resistance indexes are stable during the in vitro life cycle of *Leishmania*. Both amastigotes clones resistant to 50 μM and 100 μM DETA/NONOate conserved their resistance indexes when differentiating from amastigotes into promastigotes and then back to amastigotes in the absence of NO donor. A clone of *L. infantum* resistant to 10 μM DETA/NONOate, used in the stepwise increasing NO pressure, lost its resistance index during the differentiation processes, showing the instability of NO-resistant phenotype at low NO concentration (data not shown).

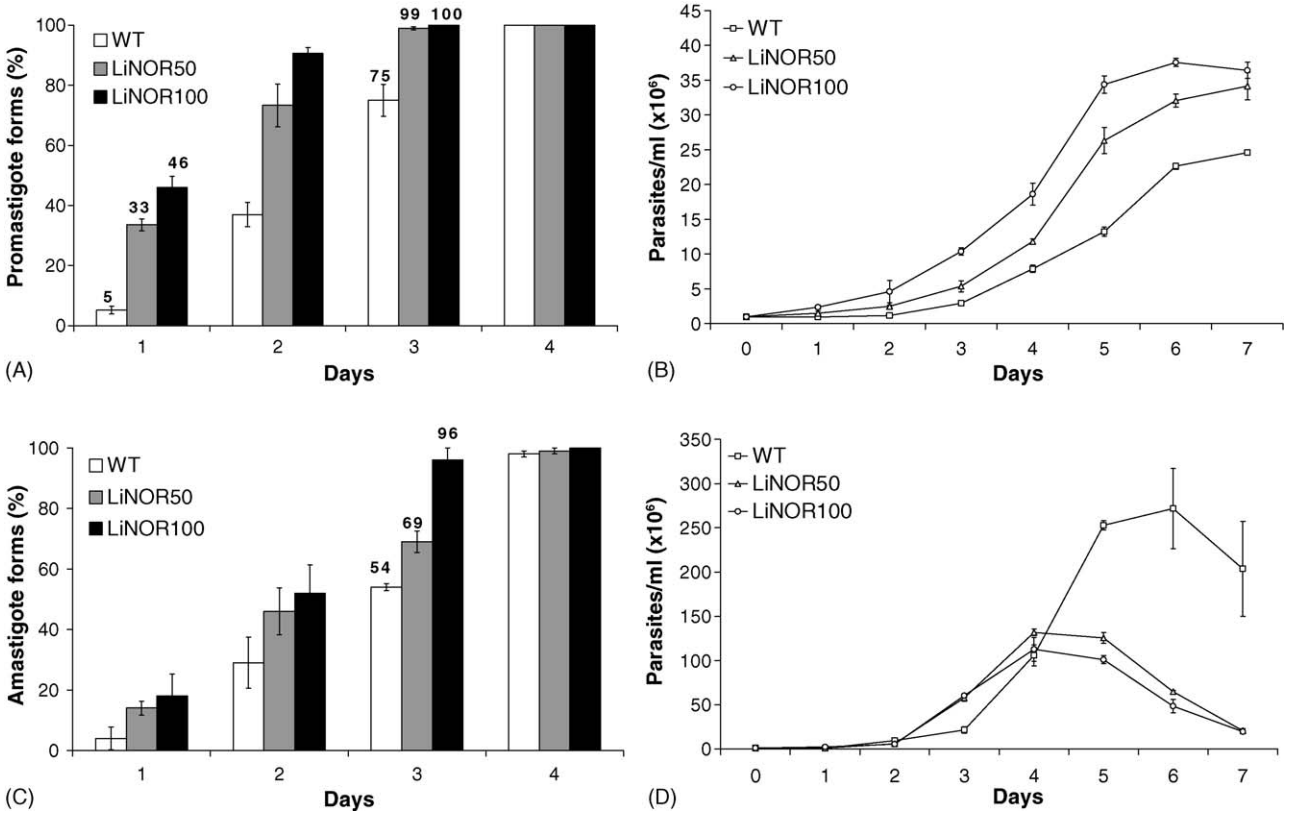

Fig. 2. *Leishmania infantum* NO-resistant amastigotes in vitro developmental cycle. Columns represent differentiation rates of wild-type and NO-resistant amastigotes into promastigotes (A) and then back into amastigotes (C). Growth curves represent the number of cells produced during the amastigote-promastigote (B) and promastigote-amastigote (D) kinetics. Differentiation experiments were performed in NO donor-free media. Results are expressed as the mean and standard deviation of three independent experiments performed in duplicate.

3.4. Susceptibility of intracellular wild-type and NO-resistant amastigotes of L. infantum to NO-mediated apoptosis

As shown in Fig. 3, LiNOR50 and LiNOR100 clones were highly resistant towards nitrogen derivatives generated ex vivo by LPS/IFN-γ-activated peritoneal macrophages of Balb/C (Fig. 3A) or C57/Black6 (Fig. 3B) mice. Within Balb/C mouse macrophages producing about 20 μM NO, when wild-type amastigotes were killed at about 90%, LiNOR50 amastigotes survived in the same way as in non-activated macrophages, and LiNOR100 amastigotes were able to proliferate 1.5 times more than in non-activated macrophages (Fig. 3A). In C57/Black6 mouse macrophages where NO levels reached to about 60 μM, LiNOR50 and LiNOR100 amastigotes were still resistant to NO action but with a lower efficacy. Wild-type amastigotes were destroyed at more than 95% whereas parasitic indexes of LiNOR50 and LiNOR100 amastigotes were only reduced by 76.1% and 42.9%, respectively (Fig. 3B).

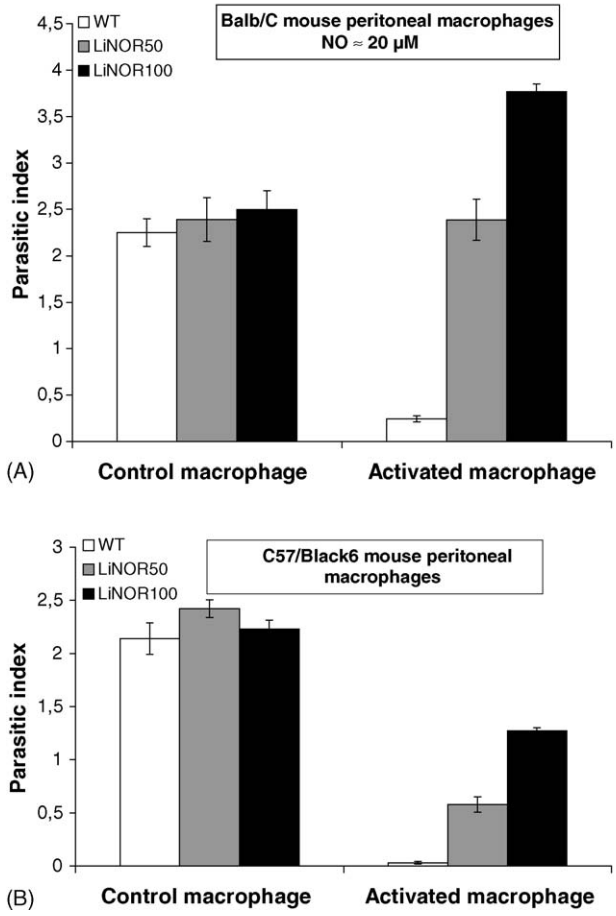

Fig. 3. NO-dependent cytotoxicity against *L. infantum* NO-resistant amastigotes in mouse activated macrophages. Balb/C (A) or C57/Black6 (B) macrophages were infected with *L. infantum* amastigotes at a parasites:cell ratio of 3:1 in RPMI 1640 medium supplemented with 10% FCS. Infected cells were treated with LPS and interferon-γ for activation and NO production. NO_2^- levels in supernatants (figured as legends) and parasite indexes were determined 48 h later. Results represent the mean and standard deviation from duplicate experiments.

The in situ TUNEL technique revealed that the nuclear DNA fragmentation of intracellular amastigotes also occurred in *L. infantum*-infected stimulated Balb/C mouse macrophages (Fig. 4). Intracellular amastigote nuclei were free of label in non-activated macrophages infected with either the wild-type clone or both NO-resistant amastigotes (Fig. 4A, C and E). Labelled amastigote nuclei could be only visualised inside macrophages infected with the wild-type clone that produced about 20 μM NO (Fig. 4B). No apoptotic nuclei were detected inside activated macrophages infected with either LiNOR50 or LiNOR100 (Fig. 4D and F). Nevertheless, some TUNEL-labelled amastigotes of LiNOR50 could be detected in C57/Black6 mouse macrophages (producing about 60 μM NO), but in less amounts than the wild-type ones, whereas no labelled LiNOR100 amastigotes were observed (data not shown).

3.5. Overexpression of metabolic enzymes in NO-resistant amastigotes

We studied here the differential expression between wild-type and NO-resistant amastigotes of different enzymes by isoenzyme assays (Fig. 5). We gave particular attention to *cis*-aconitase and glyceraldehyde-3-phosphate dehydrogenase, two metabolic enzymes in *Leishmania* parasites inhibited by NO. Six enzymatic systems were used as control and showed similar activities between pellets (day 3, day 6, data not shown) and between clones (WT, LiNOR50 and LiNOR100): glucose phosphate isomerase, glutamate oxaloacetate transaminase, isocitrate dehydrogenase (NADP+), malic enzyme, mannose-6-phosphate isomerase and nucleoside hydrolase (inosine). In the same way, superoxide dismutase, an enzyme implicated in facing the oxidative burst, was not overexpressed (Fig. 5). By contrast, *cis*-aconitase and glyceraldehyde-3-phosphate dehydrogenase were overexpressed in NO-resistant amastigotes compared to wild-type, all the more than parasites were more resistant. Interestingly, another enzyme was overexpressed by NO-resistant amastigotes with the same amount for LiNOR50 and LiNOR100, the 6-phosphogluconate dehydrogenase, which is, till date, not described as a NO target (Fig. 5).

4. Discussion

Nitric oxide (NO) has been demonstrated to be the principal effector molecule involved in the killing of *Leishmania* amastigotes by macrophages (reviewed in Nathan and Shiloh, 2000; Brunet, 2001; Colasanti et al., 2002). The findings that during experimental *Leishmania* infection, the killing of parasite can be completely reversed by L-N^G-MonoMethyl-Arginine (L-NMMA), an arginine analogue that blocks NO synthesis, and can proceed

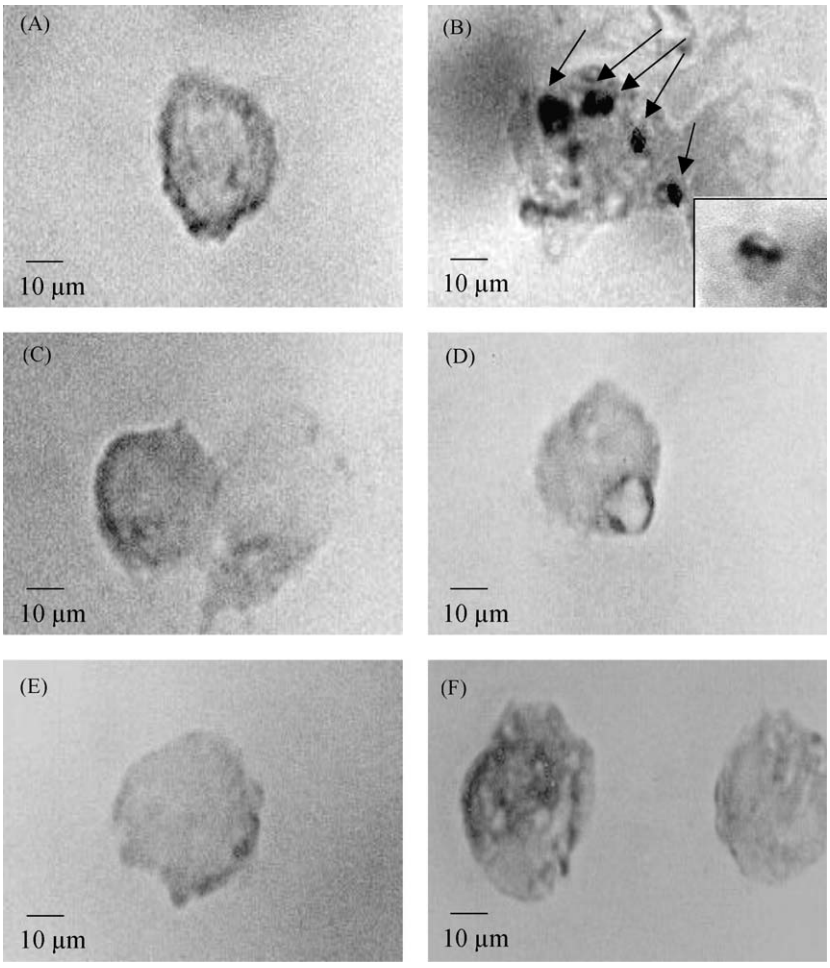

Fig. 4. In situ analysis of the NO-mediated apoptosis of *L. infantum* amastigotes in mouse macrophages. Balb/C peritoneal macrophages were infected with wild-type amastigotes (A and B) or amastigotes resistant to 50 μM (C and D) or 100 μM (E and F) DETA/NONOate at a parasites:cell ratio of 3:1 in RPMI 1640 medium supplemented with 10% FCS. Infected cells were activated with LPS or interferon-γ (B, D and F) or not (A, C and E) for 48 h. DNA fragmentation analysis was determined using the TUNEL technique and analysed under a microscope at 1000× magnification, magnification at 1600× was included in B to identify labelled amastigotes. Figure is representative of two similar experiments.

normally in a macrophage cell line deficient in the respiratory burst indicate that NO is both necessary and may be sufficient, to account for the macrophage leishmanicidal activity (Liew and Cox, 1991). Nevertheless, White et al. have demonstrated recently that NO synthase activity is not required for Solute carrier family 11a member 1 (Slc11a1; formerly natural resistance-associated macrophage protein 1)-mediated innate resistance to *Leishmania* infection (White et al., 2005). In this study, we tried to characterise the phenotypes of *Leishmania infantum* amastigotes selected in vitro for their ability to resist NO-mediated killing.

NO is a free radical which half-life (about 1 s) and physical-chemical properties prevent induction of resistance by a classical continuous NO-donor pressure protocol with *Leishmania* wild-type amastigotes. We thus started our work from *L. infantum* trivalent antimonyl-resistant amastigotes that revealed a cross-resistance to NO donors (Holzmuller et al., in press), and we succeeded for the first time in inducing different levels of NO resistance. We first determined the resistance indexes of selected amastigote clones (LiNOR50 and LiNOR100) to two NO donors and their stability throughout the in vitro parasite life cycle to characterise their NO-resistance properties (three- and five-fold resistant compared to wild-type, respectively). Interestingly, the selection of high NO resistance levels starting from weakly antimonyl-resistant amastigotes did not increase the antimony resistance level of both NO-resistant amastigote clones analysed. On the contrary, NO-resistant amastigotes lost their ability to resist trivalent antimony toxic effect. This may be a consequence of cultivation in the absence of antimonials drug pressure. Reversion of drug resistance has been demonstrated recently in K562 cells by magnetic resonance spectroscopy (Le Moyec et al., 2000). In *Leishmania*, long-term cultured parasites exihibited a loss of virulence (Segovia et al., 1992) and chemoresistant amastigotes decreased their resistance indexes when cultured for a long time in the absence of the drug (Sereno

Fig. 5. Multilocus enzyme electrophoresis (MLEE) profiles revealed with the *cis*-aconitase hydratase (ACON: E.C.4.2.1.3), glyceraldehyde-3-phosphate dehydrogenase (G3PD: E.C.1.2.1.12), 6-phosphogluconate dehydrogenase (6PGDH: EC1.1.1.44); superoxide dismutase (SOD: E.C.1.15.1.1); glucose phosphate isomerase (GPI: E.C.5.3.1.9); glutamate oxaloacetate transaminase (GOT: E.C.2.6.1.1); isocitrate dehydrogenase (NADP+) (IDH: E.C.1.1.1.42); malic enzyme (ME: E.C.1.1.1.40); mannose-6-phosphate isomerase (MPI: E.C.5.3.1.8); nucleoside hydrolase (inosine) (NHI: E.C.2.4.2.*) systems. WT: wild-type amastigotes, LiNOR50: amastigotes resistant to 50 μM DETA/NONOate, LiNOR100: amastigotes resistant to 100 μM DETA/NONOate.

et al., 1998). NO-resistant amastigotes obtained thus developed a new resistance mechanism that may share steps with the biochemical pathway involved in antimony resistance. Moreover, chemoresistance in *Leishmania* infections have been documented (Bottasso et al., 1992; Durand et al., 1998; Pal et al., 2001) and the hypothesis of becoming NO-resistant strains from antimony-resistant strains of *Leishmania* could exist in patients. Interestingly, the levels of NO resistance in vitro did not correlate exactly with resistance of NO levels generated by activated mouse macrophages. One hypothesis is that our amastigotes clones were only selected for their NO resistance whereas during macrophage activation NO and hydrogen peroxyde react to form peroxynitrite anion, which is a highly cytotoxic compound. In our experiments, generation of peroxynitrite anions could explain the partial killing of NO-resistant amastigotes inside activated macrophages. Moreover, this phenomenon seemed to be associated with the generation of elevated amounts of NO. Another hypothesis implies the Slc11a1 lysosomal protein/divalent cation transporter which plays a crucial role in resistance to *L. donovani* infection in mouse over the first 15 days without requiring NO synthase activity (White et al., 2005). In this specific hypothesis, Fe^{2+} transported by Slc11a1 might interact directly with products

of the oxydative burst to provide more potent antimicrobial radicals (Kuhn et al., 1999). Nevertheless, Slc11a1-mediated resistance to infection was expressed in resident tissue macrophages, in Kupfer cells isolated ex vivo from the livers of infected mice, or in liver, lung, or splenic tissue macrophages isolated and infected in vitro (Crocker et al., 1984, 1987; Harrington and Hormaeche, 1986) but not in less mature macrophages from the peritoneal cavity (Crocker et al., 1987), that we used in our experiments. Further interesting works will have to evaluate the potential of NO-resistant amastigotes ex vivo both in mature macrophages and in Slc11a1$^{+/+}$ versus Slc11a1$^{mt/mt}$ macrophages, and in vivo in different mouse models.

Selection for NO resistance as for drug resistance exerted a cost in term of cell metabolism. Recent works have besides shown existence of strong interactions between wild-type and chemoresistant strains of *L. amazonensis* and *L. infantum* (Agnew et al., 2001; Sereno et al., 2001). In particular, the growth and differentiation of resistant strains was reduced when they shared the same environment with a less-resistant strain. Our NO-resistant amastigotes exhibited an attenuated phase of growth as compared to wild-type in number of cells produced. The early entry in a stationary phase of growth could be considered as a resistance cost. Nevertheless, this early stationary phase of growth correlated with increased differentiation rates into promastigote forms and increased promastigote numbers. These two biological parameters argue for an elevated cell metabolism in both NO-resistant promastigote clones. In vivo, the promastigote is the parasite form that survives and develops in the sandfly. The amastigote-promastigote differentiation speed and growth rate are crucial for the generation of a transmittable parasite population. Our results demonstrated that NO-resistant amastigotes differentiated more rapidly into promastigotes, which produced more cells than the wild-type clone. These two properties endow NO-resistant mutants with a higher potential of transmission to mammalian hosts, as they can accumulate in elevated rates in the proboscis of the insect vector. Moreover, after sandfly bite, injected promastigotes resulting from NO-resistant amastigotes would differentiate again more rapidly than the wild-type ones. This resistance cost is compensated by their ability to resist to NO production and to down-regulate host macrophages activation.

Amplification of the genes encoding drug targets has been well documented in *Leishmania* chemoresistance (reviewed in Segovia, 1994; Haimeur et al., 1999; Yan et al., 2001; Guimond et al., 2003; Anacleto et al., 2003). We demonstrated in this study that resistance to NO, the natural major leishmanicidal molecule, needed overexpression of enzymes usually inhibited by nitrogen derivatives. In particular, our results have shown elevated amounts of the two metabolic enzymes, *cis*-aconitase (Lemesre et al., 1997) and glyceraldehydes-3-phosphate dehydrogenase (Mauel and Ransijn, 1997), which are inhibited by NO. Overexpression of these two NO targets could be a strategy developed by *Leishmania* to control the programmed cell death induced by NO (Holzmuller et al., 2002). Recently, NO have been shown to induce overexpression of stress proteins in *L. donovani* that protected parasites from cell death (Adhuna et al., 2000). In fact, parasitic NO molecular targets overexpression may protect the amastigotes both directly and indirectly. Directly, enzymes acted as NO scavenger and consequently detoxified the cell. Indirectly, increased glyceraldehyde-3-phosphate dehydrogenase could prevent ATP depletion and consequently cell death, by engaging anaerobic glycolysis as observed in NO-treated glucose-fed human epithelial cells (Le Goffe et al., 2002). Moreover, overexpression of *cis*-aconitase, which is considered as a two-faced protein: enzyme and iron regulatory protein (Beinert and Kennedy, 1993), would increase regulation of iron homeostasis in both the parasite and the host macrophage. In tumour cells, iron regulation played a crucial role in the cell protection from the pro-apoptotic effect of NO (Feger et al., 2001). The simultaneous addition of exogenous iron and NO donors to BCR-ABL(+) chronic myelogenous leukaemia cells reversed NO-mediated inhibition of cell growth, caspase activation and apoptosis. The quantification of intracellular iron levels in leukaemia cells indicated that NO induced an early, dose-dependent decrease in ferric iron levels (Ferry-Dumazet et al., 2002). In *Leishmania* infection, systemic iron delivery limited footpad pathology in mice infected with *L. major*. The protective effect of iron correlated with higher levels of NO synthase (Bisti et al., 2000). By limiting the pool of iron, overexpression of *cis*-aconitase could protect NO-resistant amastigotes from iron/NO injuries. In addition to *cis*-aconitase and glyceraldehyde-3-phosphate dehydrogenase, we evidenced overexpression of 6-phosphogluconate dehydrogenase (6PGDH). The 6PGDH is the third enzyme of the pentose phosphate pathway, which generates NADPH and ribulose-5-phosphate (Barret, 1997). Overexpression of 6PGDH in NO-resistant amastigotes could result in a more rapid consumption of glucose. Exhaustion of the glucose resources could explain why both LiNOR50 and LiNOR100 amastigotes showed an early entry in stationary phase of growth. One hypothesis is that the principal function of pentose phosphate pathway (PPP) in amastigote is the production of NADPH for biosynthetic uses and/or for detoxification process. The PPP plays a crucial role in the host-parasite relationship because it maintains a pool of NADPH, which is involved in protecting the parasite against oxidative stress (Dardonville et al., 2003). Dardonville et al. (2003) have tested different inhibitors of 6PGDH in *T. brucei* and showed their trypanocidal effect in vitro against the parasite. Impact of increased 6PGDH activity in NO-resistant *Leishmania* needs further investigations to determine if it acts directly in NO detoxification and by which process.

Finally, this first phenotypic study on NO-resistant amastigotes of *L. infantum* allowed us to demonstrate the overexpression of parasite molecular targets in resisting a

natural leishmanicidal compound. Overexpression of key parasitic enzymes seems to have consequences on both the developmental and cell cycles of the parasite. The potency in favouring both their transmission and their survival in infected mammalian hosts needs further investigations and discussions.

Acknowledgments

P.H. was supported by grants from the Fondation pour la Recherche Médicale (FRM Foundation). M.H. by the Centre National de la Recherche Scientifique (CNRS). D.S. and J.L.L. by the Institut de Recherche pour le Développement (IRD).

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