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Comparative proteomics profiling of a gentamicin-attenuated *Leishmania infantum* cell line identifies key changes in parasite thiol-redox metabolism

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ABSTRACT

We have previously described an attenuated line of *Leishmania infantum* (H-line), selected by culturing promastigotes *in vitro* in the presence of gentamicin. To elucidate the molecular basis for this attenuation, we undertook a comparative proteomic analysis using multiplex 2-dimensional (2D) difference gel electrophoresis. Eighteen proteins that showed significant and reproducible changes in expression were identified. Many of these were components of the thiol-redox control system in *Leishmania* and this observation, validated by Western blot, prompted us to investigate the sensitivity of the attenuated line to oxidative stress. The attenuated line was found to be significantly more susceptible to hydrogen peroxide, a change which may explain the loss of virulence. In a direct assay of trypanothione-dependent peroxidase activity, hydrogen peroxide metabolism in the H-line was significantly lower than in wild type. Furthermore, trypanothione reductase activity was significantly lower in the H-line, suggesting that gentamicin selection may result in pleiotropic affects on thiol metabolism in *Leishmania*. A putative RNA-binding protein was very strongly up-regulated in the attenuated line, suggesting a possible target for gentamicin in *Leishmania*.

1. Introduction

Leishmania infantum, the causative agent of visceral leishmaniasis in humans and dogs, is an obligate intracellular protozoan of mammalian macrophages. Control of visceral leishmaniasis in the dog can lead to a reduced prevalence of disease in associated human populations [1]. No effective vaccine is currently available against visceral leishmaniasis and control by chemotherapy is compromised because existing drugs are toxic and drug resistance is prevalent. We previously reported that a cultured attenuated line of *L. infantum*, identified as *L. infantum* H-line, was selected by culturing promastigotes *in vitro* under pressure of the aminoglycoside antibiotic gentamicin [2]. The attenuated line of *L. infantum* was phagocytosed by, but was unable to survive within, bone marrow-derived macrophages of BALB/c mice while the unselected *L. infantum* wild-type (WT) survived and multiplied within these macrophages [2]. The attenuated line failed to disseminate into visceral organs, whereas *L. infantum* WT spread into the visceral organs of infected BALB/c mice [3]. We have recently reported that *L. infantum* H-line induced no clinical signs and pathological abnormalities in dogs [3]. In addition, infection with H-line parasites elicited a Th1 response in these animals and protected against subsequent infection with wild-type parasites [4]. The attenuated

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line thus represents a potentially attractive route for the development of a vaccine.

One of the hurdles that must be overcome in order to realize this potential is to gain insight into the molecular changes that underpin selection of attenuated *Leishmania* under gentamicin pressure. Gentamicin, which is frequently added to *in vitro* cultures of *Leishmania* to control bacterial contamination [5,6], is an aminoglycoside that interacts with RNA in prokaryotic cells [7]. The precise mechanism of bactericidal activity of aminoglycosides is not fully understood, but some hypotheses include disruption of ribosomal activity by breaking up polysomes, misreading of mRNA during protein synthesis and production of abnormal or nonfunctional proteins. Aminoglycosides are less effective against eukaryotic cells, and this selectivity may reflect divergence of the translation machinery between prokaryotes and eukaryotes. Nevertheless, aminoglycosides do exhibit antileishmanial activity and one such compound, paromomycin, has recently been approved for the treatment of human leishmaniasis [8], although the mechanism of action is not understood.

Gentamicin inhibits *in vitro* growth of *Leishmania*, albeit at a higher concentration than that required to limit bacterial growth (Burchmore, unpublished observation), and we have previously shown that passage of *Leishmania* in concentrations of gentamicin that marginally inhibit growth results in the selection of cells that are avirulent [2]. Our previous data indicate that this attenuation of virulence is stable, but that attenuated parasites are able to elicit a protective immune response [9].

In the present study, a comparative proteomic analysis of *L. infantum* H-line and *L. infantum* WT, showed that expression of a number of proteins was strongly and reproducibly modulated upon attenuation. Altered expression of the redox active enzyme tryparedoxin peroxidase was prominent. We have validated this proteomic difference by Western blotting and also demonstrated that the *L. infantum* H-line express reduced tryparedoxin dependent peroxidase activity. In addition, a very strong and reproducible down regulation of a putative component of RNase P [10] was observed, a known target of aminoglycosides in eukaryotes [11]. This protein may be a target for aminoglycosides such as gentamicin and paromomycin in *Leishmania*.

2. Materials and methods

2.1. Parasites

Promastigotes of *L. infantum* JPCM5 (MCAN/ES/98/LIM-877) were cultivated in complete haemoflagellate minimal essential medium (HOMEM) (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (HI-FCS) (Labtech International) [12]. The attenuated line of *L. infantum* H-line was generated in the same medium supplemented with 10% (vol/vol) HI-FCS and 20 μg/mL gentamicin (Sigma) [2]. Briefly, amastigotes of *L. infantum* derived from the spleen of infected hamster were transferred into complete HOMEM medium and incubated at 25 °C, whereupon they differentiated to promastigotes over 72 h. These were transferred into complete HOMEM with or without gentamicin and were incubated again at 25 °C. Repeat subcultures were made every 4 days subsequently with mid- to late-log phase promastigotes. Cultures of the parasites in the absence of gentamicin were maintained in parallel to those with the antibiotic, to confirm that attenuation was not simply the result of long-term cultivation. These cultures gave rise to the wild-type parasite used in this study. Stationary phase promastigotes of H-line and wild-type parasite were harvested after 48 subpassages, a total culture period of approximately 6 months.

2.2. Sample preparation

Wild type and attenuated *L. infantum* promastigotes were grown in HOMEM with 10% HI-FCS in the absence of gentamicin. 1×10^9 stationary phase promastigotes, defined according to concentration and morphology as described previously [13], were harvested from 4 independent cultures of each line and washed three times in cold phosphate buffered saline (PBS, pH 7.4). Cell pellets were resuspended in 1 volume of difference gel electrophoresis (DIGE) lysis buffer (7 M urea, 2 M thiourea, 25 mM Tris base, 4% (w/v) 3-[(3-chola-midopropyl) dimethylammonio]-1-propanesulphonic acid (CHAPS), and 5 μL of a protease inhibitor cocktail (PI, Sigma). Samples were sonicated three times (2 s pulse with 1 min break in-between) and samples were centrifuged 10 min at 10,000×*g* to remove insoluble material. Cold acetone (four times sample volume) was added to each supernatant, the tubes vortexed and incubated for 1 h at −20 °C. The samples were centrifuged as above and the supernatant decanted. The protein pellet was washed 3 times with 80% acetone, dried briefly and resuspended in a small volume of DIGE lysis buffer (200 μL).The protein concentration was assayed in triplicate for each sample by the Bradford assay (Biorad) and the concentration adjusted to 5 mg/mL with DIGE lysis buffer. Pooled standard samples 50 μg aliquots of each sample were labelled with 400 pmol of Cy Dye for 30 min and the labelling reaction was subsequently quenched with an excess of lysine.

Labelled aliquots were combined in groups of 3, such that each mixture comprised 2 protein samples from different culture conditions (labelled with Cy3 and Cy5) and an aliquot of the pooled standard (labelled with Cy2). An additional sample was prepared that comprised 500 μg of unlabelled pooled standard (sample for preparative gel). All samples were adjusted to a final volume of 460 μL by addition of DiGE rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% DTT, 0.5% non-linear pH 3–10 IPG buffer, and 0.002% bromophenol blue), incubated at room temperature for 60 min and centrifuged 3 min at 10,000×*g*.

2.3. 2D gel electrophoresis

The supernatant from each sample mix (450 μL) was loaded onto a dehydrated non-linear pH 3–10 immobilized pH gradient (IPG) strip, overlaid with mineral oil and placed in an IPGphor (GE Healthcare). A small voltage (30 V) was applied for 12 h to permit complete rehydration of the IPG strip, then voltage was ramped to achieve isoelectric focusing (300 V, 2 h; 600 V, 2 h; 1000 V, 2 h; gradual increase to 8000 V, 3 h; 8000 V, 8 h). Immediately upon completion of this protocol, strips were transferred to equilibration buffer I (2% SDS, 50 mM Tris–Cl, pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, and 1% DTT) for 15 min and then to equilibration buffer II (2% SDS, 50 mM Tris–Cl, pH 8.8, 6 M urea, 30% glycerol,