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Leishmania (*Viannia*) *braziliensis* transfectants overexpressing the *miniexon* gene lose virulence *in vivo*

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ABSTRACT

The *miniexon* gene has a central role in the processing of polycistronic pre-mRNA of kinetoplastids. It is added to the 5′ extremity of each mRNA, supplying the 5′-capped structure to the molecule. Previous studies in *Leishmania* (*Leishmania*) *major* showed that the overexpression of the *miniexon* array attenuates the virulence of the parasite in *in vivo* assays. The results presented here extend those findings to *Viannia* subgenus. *Leishmania* (*Viannia*) *braziliensis* was transfected with a cosmid harboring a tandem array of one hundred *miniexon* gene copies and then characterized by Northern blot analysis. The overexpression of the exogenous gene was confirmed and its effect on the virulence of *L.* (*V.*) *braziliensis* was investigated in hamsters. In BALB/c mice we could not detect parasites during the course of 15 weeks of infection. In addition, hamsters infected with transfectants overexpressing the *miniexon* gene exhibited only a minor footpad swelling of late onset and failed to develop progressive lesion, these attenuated parasites could be recovered from the inoculation site 1 year after infection. The persistence of parasites in the host indicates that a stable line overexpressing the *miniexon* may be tested as live vaccine against leishmaniasis.
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1. Introduction

Leishmaniasis is a spectrum of diseases (cutaneous, mucocutaneous, and visceral) caused by parasitic protozoan of the genus *Leishmania*. This parasite goes through two basic forms: the flagellated promastigote, which proliferates in the gut of a sand fly and is transmitted to the mammalian host by the bite of the insect vector, and the amastigote form, which replicates and survives within the phagolysosome of the host macrophage [1]. The variety of biological novelties detected in this ancient eukaryote and its medical relevance have led several research groups to investigate the biology and molecular processes it undergoes. The parasite has unusual mechanisms for the flow and control of gene expression, such as polycistronic transcription, *trans*-splicing and RNA editing [reviewed by 2,3]. Messenger RNA synthesis in kinetoplastids protozoa involves the addition of a spliced leader (a 39-nucleotide capped miniexon) sequence to most, if not all, maturing mRNA. The spliced leader is initially transcribed as a 140-nucleotide miniexon-derived RNA [4] and the transfer of the 5′-capped 39-nucleotide miniexon to the 5′-end of each pre-mRNA occurs by a bimolecular *trans*-splicing reaction.

The *miniexon* gene (ME) is organized in a tandem array in the genome of all trypanosomatids and its sequence and extension varies among species [4–6]. Nevertheless, its overall structure is conserved, containing two functional domains: the exon, highly conserved, and the intron, which needs a conserved secondary structure to be functional. These functional domains are followed by a variable untranscribed region. In *Leishmania major* the array resides on chromosome 2 and the number of repeats, which are 455 bp long, varies among different strains [4–9].

The *miniexon* gene has a central role in the RNA processing reaction of kinetoplastids protozoa. It is required for RNA maturation, transcript stability and initiation of translation. The parasite is unable to survive in the absence of miniexon transcription [10–12]. Iovannisci and Beverley associated expansion of the miniexon array with growth advantage of promastigotes *in vitro* [13]. Samaras and Spithill showed that an avirulent *L.* (*L.*) *major* clone presented a larger chromosome 2 and suggested that its size polymorphism could be due to an expansion of the miniexon locus [11]. On the other hand, a few *L.* (*L.*) *major*

Abbreviations: SL RNA, Spliced leader RNA; bp, Base pairs; nt, Nucleotides; kb, Kilobase pairs; HEPES, N-2 hydroxyethylpiperazine–N%-2-ethanesulfonic acid; ME, Miniexon; LNs, Lymph nodes.

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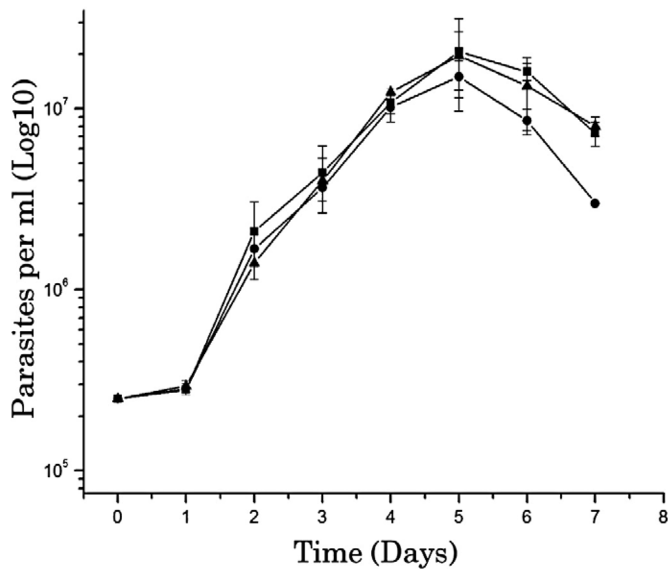

Fig. 1. Growth curve of *L.* (*V.*) *braziliensis* parental and transfectant lines. Parental line was grown in M199 supplied with 2% of human urine and the transfectants were cultured with 120 µg/ml of hygromycin B. The number of parasites was estimated using a hemocytometer. *L.* (*V.*) *braziliensis* parental line (square); Lb [*cLHYG*^{120}] (triangle); Lb [*cLHYG*^{120}*ME*] (circle).

virulent lines, such as Friedlin and LV39, bear an amplified version of one or both alleles of chromosome 2 [14]. Antoniazi et al. demonstrated that virulent *L.* (*L.*) *major* lines lose virulence in *in vivo* infection assays after transfection of a recombinant cosmid bearing only the *miniexon* gene array. Overexpressors "cured" of the circular molecule reverted to the virulent phenotype, which confirms a positive correlation between overexpression of the miniexon and loss of virulence [15].

In this study we show that hamsters infected with a *Leishmania* (*V.*) *braziliensis* strain overexpressing the *L.* (*L.*) *major* miniexon array (*cLHYG ME*) does not develop progressive lesions. The remarkable phenomenon of virulence attenuation observed in *L.* (*V.*) *braziliensis* indicates that overexpression of the *miniexon* gene affects the outcome of infection.

2. Materials and methods

2.1. Cell lines, culture conditions and transfection

Clonal virulent *L.* (*V.*) *braziliensis* H3227 (MHOM/BR/94/H-3227) was used to host the miniexon recombinant as an extrachromosomal molecule. Cells were grown in M199 medium supplemented with 10% fetal bovine serum, 2% human urine, 100 mM adenine, 10 mg/ml hemin, 40 mM HEPES (pH 7.4), 50 units/ml penicillin and 50 mg/ml streptomycin. Cells grown to late log phase were transfected by electroporation (500 µF and voltage 2.25 kV/cm) and stable transfectants were selected in semi-solid medium (32 µg/ml hygromycin B), as described previously by Kapler et al. [16]. Transfectants were named according to Clayton et al. [17] and drug concentration used in liquid culture (µg/ml) is indicated by superscript. The control line carrying the cosmid with no insert and ME overexpressor bearing one hundred copies of the *L.* (*L.*) *major* ME array were named Lb [*cLHYG*] and Lb [*cLHYG ME*] respectively. We used the parasites cultured in liquid medium at drug concentration of 12 and 120 µg/ml hygromycin B in order to compare the effect of heterologous ME overexpression under two different drug pressure conditions.

2.2. Total RNA preparation

Total RNA was prepared with Trizol reagent (Invitrogen). Samples were fractionated by either denaturing 1% agarose gel or denaturing 12% polyacrylamide gel electrophoresis, blotted to nylon membranes (GeneScreen Plus, Dupont) and hybridized following standard methods [18].

2.3. DNA labeling

A PCR-amplified fragment of the *hygromycin phosphotransferase* gene (*hyg*) and a 455 bp ME fragment derived from a Not I digestion of 32D05 (the cosmid bearing the miniexon array) [15], were used as templates for a random priming labeling reaction [19,20] with [α-^{32}P] dCTP (GE Healthcare, Piscataway, PA, USA) to produce probes for Northern experiments.

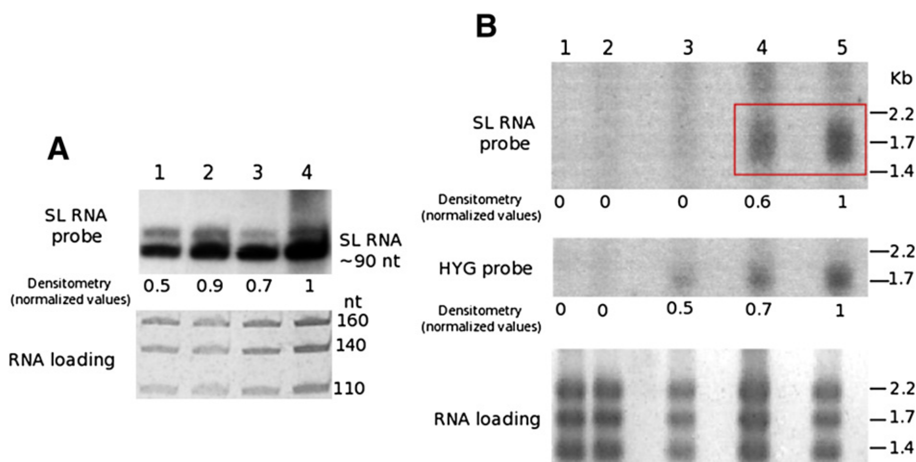

Fig. 2. Transcript levels of SL RNA and HYG genes and evaluation of trans-spliced RNA levels in promastigotes. A) Trizol-extracted RNA from promastigotes maintained in axenic culture of: (1) Lb [*cLHYG*^{120}] log phase, (2) Lb [*cLHYG*^{120}*ME*] log phase, (3) Lb [*cLHYG*^{120}] stationary phase and (4) Lb [*cLHYG ME*^{120}] stationary phase. The RNA was resolved in a 12% polyacrylamide gel. Hybridization probe was a fragment of 455 bp derived from 32D05 cosmid digested with Not I corresponding to one entire copy of *miniexon* gene. Ribosomal RNA was used to normalize the sample loading. The numbers at the bottom of each panel are relative to the normalized densitometry values. B) Trizol-extracted RNA from promastigotes maintained in axenic culture of: (1) *L.* (*V.*) *braziliensis* parental, (2) Lb [*cLHYG*^{12}], (3) *Lb* [*cLHYG*^{120}] and (4) Lb [*cLHYG*^{12}*ME*]. (5) Lb [*cLHYG*^{120}*ME*]. The RNA was resolved in agarose gel 1.0%. Hybridization probes: HYG, a PCR fragment of 1 kb containing the entire hygromycin phosphotransferase coding region; ME, a fragment of 455 bp derived from 32D05 cosmid digested with Not I corresponding to one entire copy of *miniexon* gene. Ribosomal RNA was used as a sample loading control. The numbers at the bottom of each panel are relative to the normalized densitometry values. All quantification was conducted by ImageJ software and the hybridization signals were subtracted from background level and normalized with sample loading.

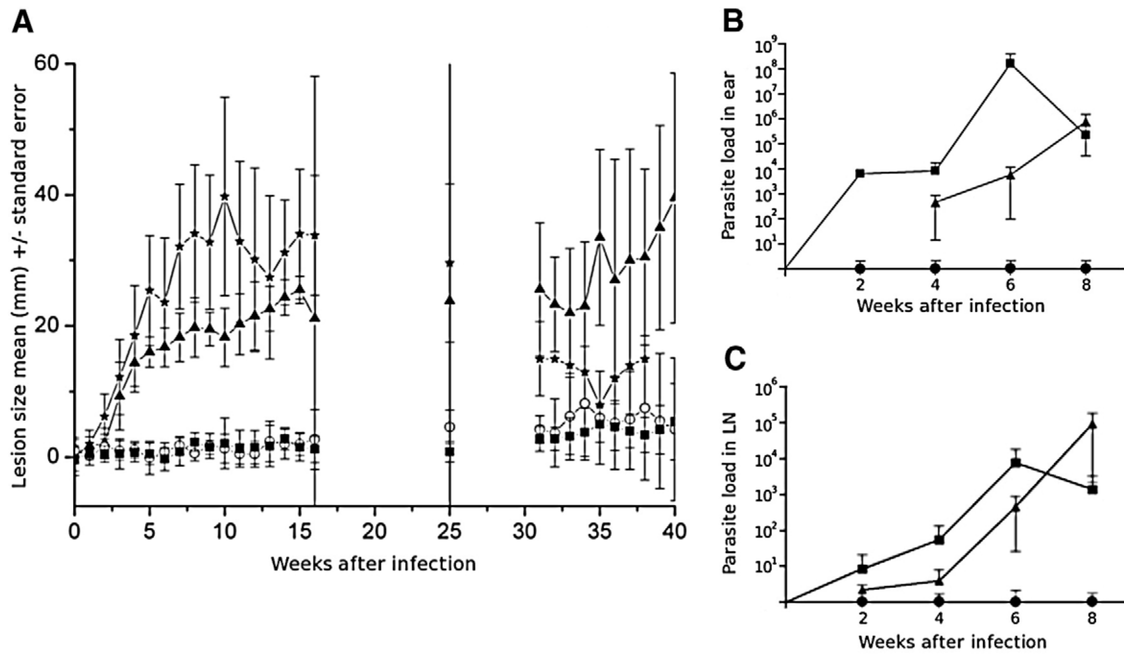

Fig. 3. Course of infection in hamsters (*M. auratus*) and parasite load estimate in BALB/c mice after intradermal inoculation with *L.* (*V.*) *braziliensis* parental and transfectant lines. (A) Stationary phase of axenic promastigotes was subcutaneously injected in the hind footpad of hamsters and lesion development was monitored by measurement of footpad thickness with a Mitutuyo digital caliper. Each point represents the mean lesion size (±standard error) of ten animals per group. *L.* (*V.*) *braziliensis* parental line (triangle); Lb [*cLHYG*^{12}] (star); Lb [*cLHYG*^{12}*ME*] (open circle) and Lb [*cLHYG*^{120}*ME*] (square). (B and C) Mice were infected with 1×10^5 *L.* (*V.*) *braziliensis* promastigotes per ear. Limiting dilution assay was used to determine ear (B) and draining lymph node (C) parasite loads at 2, 4, 6 and 8 weeks post-infection. Data represent the mean ± standard error in four independent experiments, each performed on six mice. *L.* (*V.*) *braziliensis* parental line (square), Lb [*cLHYG*^{120}] (triangle), Lb [*cLHYG*^{120}*ME*] (circle).

2.4. Animals, parasite inoculation and lesion measurement

Hamsters were obtained from an animal facility in the Medical School (FMRP-USP, Ribeirão Preto, SP, Brazil). Stationary-phase *L.* (*V.*) *braziliensis* (10^7 viable parasites in 25 µl of saline) promastigotes were inoculated subcutaneously into the right hind footpads of hamsters ($n=10$ per experiment). Lesion progression was recorded once a week by measuring footpad swelling (dorsal to plantar axis) with a vernier caliper, using the non-infected contra-lateral rear footpad as a control. One year after infection, footpads from five hamsters were harvested for RNA extraction and Northern analysis. Female BALB/c mice (4–8 weeks old) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB-UNICAMP, Campinas, SP, Brazil). Stationary-phase promastigotes (10^5 viable parasites in 10 µl of saline) were inoculated into the right ear dermis of age-matched BALB/c mice using a 27.5-gauge needle ($n=5$ per experiment) [25]. Lesion size was monitored weekly for 10 weeks with a digital caliper (Thomas Scientific, Swedesboro, NJ).

2.5. Parasite load estimate

Infected ears and retromaxillar draining lymph nodes (LNs) were aseptically excised from BALB/c mice at 2, 4, 6 and 8 weeks post-infection and homogenized in Schneider's medium (Sigma Chemical Co., St. Louis, MO). The homogenates were serially diluted in Schneider's medium supplemented with 10% fetal bovine serum and 2% human urine, and distributed into 96-well plates containing biphasic blood agar (Novy-Nicolle-McNeal) medium. Parasite load was determined using a quantitative limiting dilution assay as described previously [21].

2.6. Culture and infection of J774 macrophages

Cultures of J774 macrophages were maintained in RPMI medium supplemented with 10% of bovine fetal serum at 37 °C in 5% CO_2. J774 cells were infected with *L.* (*V.*) *braziliensis* in a ratio of 1:5 for 3 h at room temperature.

2.7. Electron microscopy

For transmission electron microscopy, 2×10^6 J774 macrophages were pelleted at 1000 ×g, washed twice in PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and then fixed in 2% glutaraldehyde

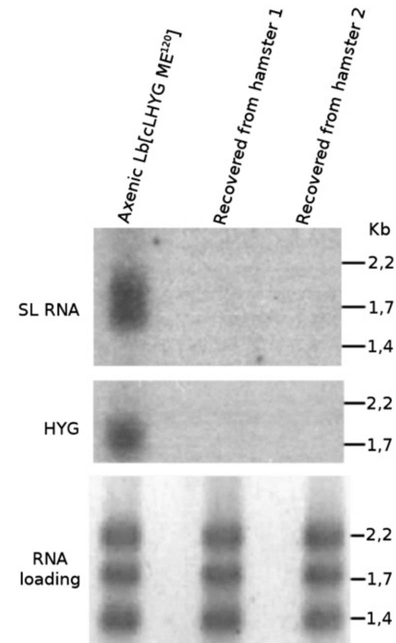

Fig. 4. Northern blotting analysis of parasites recovered from *M. auratus* footpads: lane 1) Trizol-extracted RNA from axenic transfectant Lb [*cLHYG*^{120}*ME*]; lanes 2 and 3) Trizol-extracted RNA from promastigotes recently differentiated from parasites recovered from two different hamsters after 1 year of infection. Hybridization probes: HYG, a PCR fragment of 1 kb containing the entire hygromycin phosphotransferase coding region; ME, a fragment of 455 bp derived from the 32D05 cosmid digested with Not I which corresponds to a entire copy of *miniexon* gene. Ribosomal RNA was used as a sample loading control.

(Ladd Research Industries, Burlington, VT), 2% paraformaldehyde (Ladd) in 0.1 M cacodylate buffer, pH 7.4; containing 0.05% $CaCl_2$, for 2 h at room temperature. Cells were washed twice in 0.1 cacodylate buffer, pelleted and maintained in 0.1 M cacodylate buffer, pH 7.4; containing 5% sucrose. Cells were post-fixed in 2% OsO_4 (Electron Microscopy Sciences, Hatfield, Pennsylvania) for 1 h at room temperature, rinsed in distilled water, dehydrated through a graded series of ethanol, rinsed in acetone, and embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, Pennsylvania). Sections were examined in a Phillips 208 Electron Microscope. All images were prepared for publication using The Gimp (Free Software Foundation, Boston, Massachusetts).

3. Results

3.1. L. (V.) braziliensis transfectants: growth behavior of axenic promastigotes

Axenic cultures of promastigotes were grown to evaluate the proliferative pattern of miniexon overexpressor as compared to control lines. All the transfectants, control lines and miniexon overexpressor exhibited growth rates and final cell densities similar to the parental *L.* (*V.*) *braziliensis* line (H3227) (Fig. 1). Growth behavior of these clones did not change after ten passages in axenic culture or after recovery from infected animals in the tenth week after infection (data not shown).

3.2. Molecular characterization of L. (V.) braziliensis transfectants

To investigate the correlation between miniexon transcript level and virulence pattern we transfected *L.* (*V.*) *braziliensis* (H3227) with [*cLHYG ME*]. Clones resistant to hygromycin B were isolated and the presence of the circular extrachromosomal molecule was confirmed by PFGE (Pulse Field Gel Electrophoresis), followed by Southern hybridization using a *miniexon* gene fragment as probe (data not shown). We assessed the exogenous miniexon transcript level by Northern blotting analysis. RNA from Lb [*cLHYG ME*] and Lb [*cLHYG*] transfectants was extracted in exponential and stationary growth phases, fractionated in 12% polyacrylamide gel and probed with the miniexon fragment. The results show that Lb [*cLHYG ME*] transfectants contain between 30 and 40% more miniexon transcript (90 nucleotides long) than the control line, Lb [*cLHYG*] in both growth phases examined (Fig. 2A). In addition, the Lb [*cLHYG ME*] line presented a strong signal that appears as a clump in the region corresponding to 1.5–2 kb RNA molecules, a size range in which most of the *Leishmania* mRNA resides (Fig. 2B, lanes 4 and 5). In the same region of the blot no signal was detectable in any of the control lines, the parental line, Lb [$cLHYG^{12}$] and Lb [$cLHYG^{120}$] under the same conditions (Fig. 2B, lanes 1, 2 and 3). The Northern hybridization with the *hyg* probe demonstrates that the transfectants selected harbor the cosmid and that the transcription of the foreign molecule is proportional to drug pressure. The normalized densitometry values at the bottom of each panel reveal that the transgene *hyg* and the *miniexon* gene follow the same relative expression pattern. This suggests that the increase in the

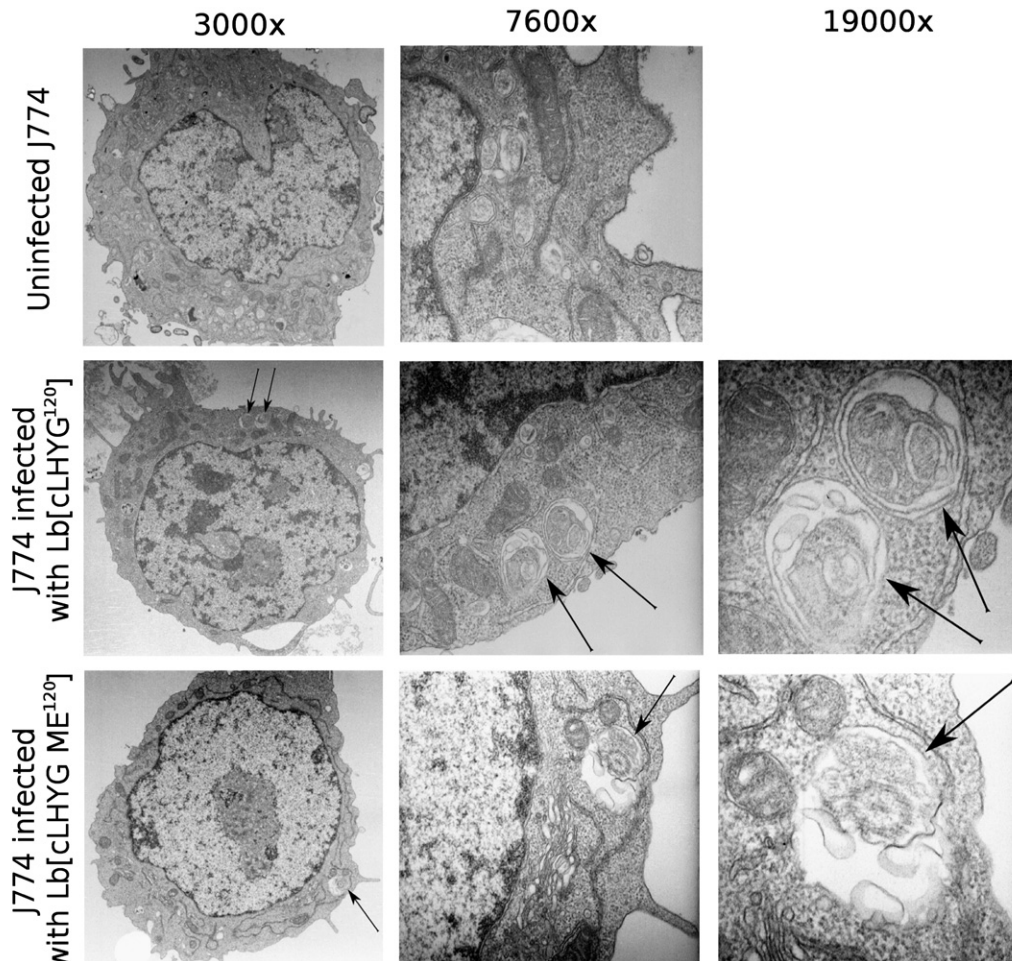

Fig. 5. Ultrastructural and molecular evaluation of invasion process. Electron microscopy of J774 macrophages infected *in vitro* with transfectant overexpressing the *miniexon* gene and the transfectant control. The arrows indicate the amastigotes inside parasitophorous vacuoles (3000×, 7600× and 19,000×).

population of *trans*-spliced transcripts detectable in the Lb [*cLHYG ME*] is caused by the exogenous miniexon molecule. Northern blotting analyses indicate that the ME overexpressor presents an enhanced miniexon transcript level and that total *trans*-spliced mRNA levels are increased.

3.3. Course of experimental leishmaniasis reveals that transfectants overexpressing ME RNA lose virulence in vivo

The transfectants were cultured in 12 and 120 µg/ml hygromycin B in order to observe the effect of miniexon transcript surplus on the virulence. Stationary-phase promastigotes (1×10^7) were injected subcutaneously in the hind footpad of ten hamsters. The *L.* (*V.*) *braziliensis* parental line (H3227) and the control transfectant, Lb [*cLHYG*], were used as negative control lines. Lesion development was monitored for 1 year. A dissimilar increase in footpad swelling was observed when comparing the groups for 40 weeks after infection. Hamsters inoculated with the parental line (H3227) showed progressive growth of cutaneous lesions up to 15 weeks. Lb [*cLHYG*] induced the development of even more severe footpad lesions than the control Lb. Therefore, neither [*cLHYG*] nor hygromycin B is involved in the loss of virulence. On the other hand, hamsters inoculated with the Lb [*cLHYG ME*] transfectant exhibited only minor footpad swelling and failed to develop progressive infection (Fig. 3A). This phenomenon was observed after inoculation with Lb [*cLHYG ME*] cultured at either 12 or 120 µg/ml hygromycin B before infection in animal models (Fig. 3A).

We also used a novel experimental model of infection to study the ability of *L.* (*V.*) *braziliensis* parental (H3227) and transfectant lines (Lb [$cLHYG^{120}ME$] and Lb [$cLHYG^{120}$]) to induce lesion development in BALB/c mice. Animals were infected in ear dermis with 10^5 parasites; the wild type H3227 showed a peak of parasite load 6 weeks after infection, both at the inoculation site (Fig. 3B) and in draining lymph nodes (Fig. 3C). A similar pattern was observed in animals infected with Lb [$cLHYG^{120}$]. However, no parasites were detected in mice infected with Lb [$cLHYG^{120}ME$] throughout the entire period of the experiment (Fig. 3B and C), which supports the correlation between miniexon transcript excess and virulence loss. These data confirm previous findings [15] and extend the positive correlation between extrachromosomal overexpression of the *miniexon* gene and virulence attenuation to *L.* (*V.*) *braziliensis*.

3.4. Molecular analysis of parasites recovered from animals confirms the correlation between miniexon transcript excess and attenuation

Infected hamsters were monitored for 1 year. After this period two out of ten animals from the Lb [*cLHYG ME*] group presented a small lesion on the infected footpad. This finding led us to investigate a possible ME transcript level decrease in parasites recovered from these animals. Amastigotes were rescued from the footpads and total RNA was extracted from recently differentiated promastigotes. The RNA was fractionated in denaturing agarose gels and transferred to nylon membranes for Northern blot analysis. We observed that parasites recovered from these lesions no longer overexpressed the hygromycin phosphotransferase or the exogenous *miniexon* gene (Fig. 4). This result confirms that the excess of ME transcripts negatively affects the virulence of *Leishmania* during the infection of a mammalian host. In addition, we demonstrated that the miniexon overexpressor transfectant maintains its ability to infect and differentiate within J774 macrophages *in vitro* (Fig. 5).

4. Discussion

In this study we report the influence of miniexon expression levels on virulence patterns of *L.* (*V.*) *braziliensis*. The availability of a cosmid bearing 100 repeats of the *L.* (*L.*) *major miniexon* gene and well-established infection protocols, together with our ability to stably introduce cosmids into *L.* (*V.*) *braziliensis*, made it possible to correlate the artificial overexpression of ME with virulence decrease. We worked with cloned transfectants, selected as colonies from solid medium supplemented with hygromycin B. Two animal models with different genetic and immune backgrounds were infected with these clones, and two modes of infection were used (subcutaneous and intradermal injection). Working with large groups of animals and independent experiments, we observed the attenuation of *L.* (*V.*) *braziliensis* ME overexpressor in all cases.

This investigation extends a previous study by our group with similar results in *L.* (*L.*) *major* [15]. Therefore, the observed phenomenon occurs within two distinct subgenera. Virulence attenuation is more noticeable in *L.* (*V.*) *braziliensis* transfectants, as no lesion is observed even using one tenth of the drug concentration necessary to attenuate *L.* (*L.*) *major* [*cLHYG ME*]. Previous and present data allow us to associate miniexon overexpression with loss of virulence.

Conflicting data have been reported by Zhang and Matlashewski [22]. They have recently described an increased virulence of *L.* (*L.*) *major* transfectants harboring either a *L.* (*L.*) *donovani miniexon* gene array or the [*cLHYG ME*] construct. A drawback of their study is that a population of resistant cells was used instead of clonal lines. Although these authors recovered virulent transfectants from infected animals, they did not report whether the loss of the extrachromosomal molecule leads to the reversion of the phenotype, a crucial experimental step in order to establish the correlation between the transfected molecule and virulence. Therefore, the experimental design adopted by Zhang and Matlashewski [22] does not allow exclusion of a virulence variation unrelated to the presence of ME cosmid. In addition, it is known that variability of parasite fitness can occur naturally due to unknown factors, leading to an apparently spontaneous virulence reversion. This phenomenon has been described by different authors and represents an obstacle to the use of naturally avirulent lines as live vaccines [15,23]. The dynamics of virulence in these parasites is complex and this poorly understood issue could be the result of a combination of factors, including epigenetic ones [24].

Our previous data from the *L.* (*L.*) *major* ME overexpressor showed that the “cure” of the extrachromosomal molecule leads to a reversion to the parental phenotype. This study with *L.* (*V.*) *braziliensis* has added relevant data to better understand the attenuation induced by overexpression of the miniexon. The persistence of the parasite and the long-lasting effect of ME overexpression are important features revealed here. We have demonstrated that the *L.* (*V.*) *braziliensis* ME transfectant, rescued from hamsters after 1 year of infection showed undetectable levels of expression of the exogenous genes. Nevertheless, these parasites could grow in axenic culture supplemented with hygromycin B, which suggests that the cosmid is present in at least some of the cells of the population. The subtle clinical manifestation induced by this attenuated parasite after more than 40 weeks of hamster infection shows that Lb [$cLHYG^{120}ME$] does not mimic the virulence pattern of the parental line. Therefore, the parasite persistence in infected hamsters and the successful infection of J774 macrophages supports the hypothesis that the inability of the mutant to cause disease is due to an impairment of amastigotes to proliferate rather than a failure to invade the host cell. Although an impairment of Lb [*cLHYG ME*] to infect BALB/c cells could be an explanation for the undetectable levels of parasites in these animals this is not supported by our current and previous data [15].

Leishmania mutants overexpressing the *miniexon* gene could be a suitable model for further studies on host–parasite interactions and could be useful to better understand some aspects of virulence and the parasite's needs to proliferate within macrophages.

Presented results suggest that the miniexon mutant has an increased activity of trans-splicing which leads to high levels of trans-spliced transcripts. Presented data associated with the fact that high levels of drug pressure lead to an increased expression of all the

genes of recombinant cosmids [26] indicate that the *miniexon* gene is overexpressed in this mutant. We may postulate that the probable overexpression of the exogenous miniexon may lead to an imbalance of translated product levels, which should be down-regulated in amastigotes and crucial for the proliferation of this intracellular form. The imbalance generated is incompatible with the parasite's intracellular life in the hostile phagolysosome. Further studies should attempt to answer these questions.

We have demonstrated that *L.* (*V.*) *braziliensis* transfectants overexpressing the spliced leader RNA lose their original virulent pattern, similarly to what had been previously observed in *L.* (*L.*) *major* ME overexpressors. Besides extending previous findings to another subgenus, we have shown here that these attenuated parasites are persistent within the host. These mutants may be important for the generation of safe vaccines and are also useful tools for the study of the control of gene expression routes in *Leishmania*.

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