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A 245 kb mini-chromosome impacts on *Leishmania braziliensis* infection and survival

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ABSTRACT

Leishmania (V.) *braziliensis*, the causative agent of mucocutaneous leishmaniasis in the New World, may present an LD1 type genomic amplification that appears as a small 245 kb linear chromosome, and is not clearly associated to the presence of a selection agent. A *bt1* gene, codifying for a biopterin transporter protein, was identified in this small chromosome. *Leishmania* are auxotrophic for pterins and one of the proposed explanations for the appearance of this amplification is the improvement of biopterin capture by the parasite. We analyzed some biological aspects of two lineages of *L. braziliensis* strain M2903, with and without the small amplified chromosome. We showed differences in infectivity of these lineages, in macrophages and the insect vector *Lutzomyia longipalpis*, as well as in the uptake and metabolization of intermediates of the *Leishmania* biopterin salvage pathway. Our results suggest that the genomic amplification favors survival due to improved biopterin capture and at the same time hinders the infective capability, suggesting that within a population different parasites can perform different roles.

Introduction

Leishmania parasites are the causative agents of leishmaniasis, a serious public health problem that strikes approximately 15 million people mostly in tropical and subtropical regions. The various species of these parasites cause two clinical forms of disease, visceral and dermotropic. This last form can take the cutaneous and muco-cutaneous manifestations, of which *Leishmania braziliensis* is one of the causative agents [1].

The genome of *Leishmania* is distributed among 34–36 chromosomes that range from 150 kb to more than 2 Mb and chromosome size polymorphism is observed among species and strains. Additional genetic material can be found in multicopy small chromosomes, which appear as a result of genetic amplifications. These amplifications can arise in the presence or absence of drug pressure, and can be either linear or circular [2]. The best characterized spontaneous amplifications belong to the LD1 amplicon family [3], a 27.5 kb DNA sequence derived from chromosome 35 that appears as a circular, repeated and inverted episomal element in *Leishmania infantum* [4] and in other *Leishmania* specie [5–7]. *L. braziliensis* M2903 also presents an amplification belonging to this family, in the form of a small linear 245 Kb chromosome [7–9].

Sequence analysis of this amplicon in *L. infantum* and *Leishmania major* revealed the presence of many open reading frames (ORFs). Among these sequences ORF G codifies a biopterin transporting protein and is present in all *Leishmania* LD1s [10,11], including *L. braziliensis* M2903 [12].

Considering that *Leishmania* are auxotrophic for pteridines, the presence of this gene was considered to be the possible force driving the appearance of LD1 type amplifications [13]. Confirming the *Leishmania* complete dependence for exogenous sources of pteridines, the elimination of the three genes encoding for the BT1 biopterin transporter in *Leishmania donovani* created mutants incapable of growing in non-supplemented medium [14]. To overcome this deficiency, parasites have evolved a salvage pathway which utilizes proteins that capture biopterin (BT1) and folate (FT1). In the cell these metabolites are enzymatically reduced to tetrahydrobiopterin (BH4) and tetrahydrofolate (FH4). *Leishmania* are resistant to anti-folates, and the deletion of the main folate (F) transporter in *Leishmania tarentolae* is compensated by the overexpression of BT1, the biopterin transporter that also captures folate [15]. This pathway can be regulated by the higher expression of genes codifying

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for the transporter proteins, or by the inhibition/stimulation of the enzymes dihydrofolate reductase-thymidylate synthase (DHFR-TS) and pteridine reductase1 (PTR1).

The function of pteridines in *Leishmania* growth is still not clear. It is known that they reduce the need for folates in *Crithidia fasciculata* [16] and that they might be involved in their synthesis [17], since cells can grow in folate deficient medium that contains biopterin [18].

We took advantage of the availability of two *L. braziliensis* M2903 lineages, one containing and one lacking the linear chromosome amplification of 245 kb, to evaluate biochemical and biological differences potentially caused by this difference. We studied the infectivity of the two lineages in the invertebrate vector and in macrophages, the cells involved in the successful infection of the mammalian host. We also studied the possible biochemical advantages derived from the overexpression of the *bt1* gene, the increased resistance to methotrexate, known inhibitor of DHFR-TS, and the reversion of this inhibition in the presence of substrate, reduced or not.

Materials and methods

Parasites. L. braziliensis M2903 (MHOM/BR/75/M2903) containing the 245 kb chromosome (here called M2903(+)) were obtained from Dr. Gabriel Grimaldi (Fiocruz); the lineage lacking the small chromosome (here called M2903(−)) was obtained from Dr. Diane McMahon-Pratt (Yale University). These were maintained at 25 °C in 199 medium supplemented with 10% fetal bovine serum [19].

Macrophage infection. Murine macrophages lineage J774G.8 kept in RPMI medium with 10% fetal bovine serum were submitted to 10,000 rads (^{137}Cesium). After cell viability verification using trypan blue, the concentration was adjusted to 10^5 cells per well. Parasites were grown to stationary phase, washed twice for 15 min with RPMI medium without serum, counted and adjusted to 20 parasites per macrophage. Infection was done in 24 well plates containing glass coverslips dipped in FBS, in RPMI medium with 10% FBS, and evaluated after 3, 6 and 24 h. The glass slips were stained by the Leishman method [20], observed with an Axiolab (Zeiss) microscope and the number of amastigotes per cell, as well the percentage of infected macrophages, were evaluated.

Artificial infection in sandflies. Three groups of 50 laboratory-reared *Lutzomyia longipalpis* females 5–6 days old from Lapinha Cave (Brazil) were artificially infected as described by Ward et al. [21] with 10^7 promastigotes of either lineage per milliliter in human blood. Subsequently, the flies were maintained at 26 ± 1 °C, fed on saturated sucrose solution *ad libitum*. Seven days after the infectious feeding, the females were anaesthetized at −15 °C by 2–3 min, then shaken in a vial containing phosphate buffered saline (PBS) and dissected. The fresh guts were examined under phase contrast illumination with 40× augmentation for the presence of parasites.

Growth curves of both lineages of L. braziliensis in the presence of pteridine pathway metabolites. Both lineages of *L. braziliensis* were grown in 199 medium with 10% fetal bovine serum to the end of logarithmic growth, and transferred to the same medium containing various concentrations of biopterin (0 and 15 ng/mL), folate (0 and 10 ng/mL), tetrahydropterin (0 and 0.1 μg/mL) and methotrexate (0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0, 256.0, 512.0, 1024.0 nM). Initial inoculum was 10^5 parasites/mL and growth was evaluated counting cells in a Neubauer chamber. Growth results were compared by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Infectivity in macrophages

Our previous experiments determined that infection of J774.G8 murine macrophages by *L. braziliensis* was most efficient in a proportion of 20 parasites/cell. Until 3 h after infection we observed no internalization of promastigotes, with 67% of macrophages exposed to lineage M2903(+) and 84% of those exposed to lineage M2903(−) presenting adhered parasites. After 6 h of infection, 93% of the macrophages were infected with 12–15 M2903(−) amastigotes/cell, versus 35% infected with approximately 7 M2903(+) amastigotes/cell. After 24 h, we verified that isolate M2903(−) still infected 72% of macrophages, while lineage M2903(+) infected only 19% of the cells. These results are expressed as infectivity indexes (Fig. 1), calculated by multiplying the percentage of infected macrophages by the number of amastigotes present per cell.

Infectivity in sandflies

No infection was seen in female *L. longipalpis* 7 days after feeding on blood containing 10^7 promastigotes/mL from isolate M2903(+). Isolate M2903(−) infected 20% of the females, with a distribution in the gut of *L. longipalpis* compatible with the pattern of infection expected from *Leishmania* from the sub-genus *Viannia* (Table 1). Infected females had 10–30 parasites.

Growth of the two L. braziliensis lineages in the presence of pteridine metabolites

The addition of biopterin to culture medium showed a stimulatory effect of approximately 20% over the growth of isolate M2903(−), and 110% over isolate M2903(+) (Fig. 2). The proliferation after addition of tetrahydrobiopterin to the medium showed an increase of up to 95% in M2903(+) and 25% in relation to the control experiment (Fig. 2).

In the presence of low concentrations of folate (10 ng/mL) a different result was observed; while M2903(−) did not show any growth stimulation in relation to the control, growth of M2903(+) was stimulated by approximately 60%. The effect of folate incorporation to the medium already containing biopterin or tetrahydrobiopterin was also studied. Lineage M2903(−), which in the presence of folate alone was not stimulated, presented a discrete stimulation when biopterin or tetrahydrobiopterin were added. On the other hand, the presence of tetrahydrobiopterin induced a discrete stimulation of growth of lineage M2903(+) (Fig. 2).

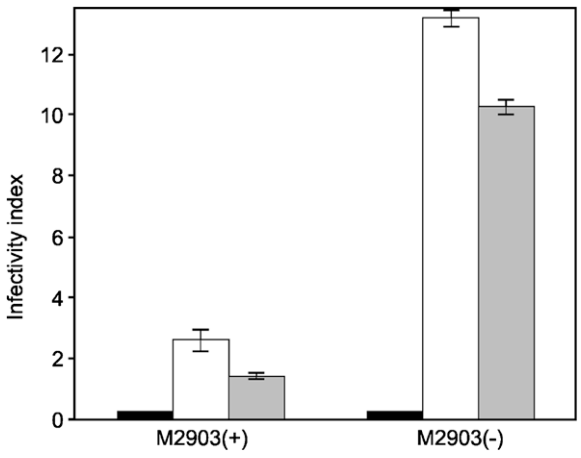

Fig. 1. Infection of murine macrophage J774.8 with *L. braziliensis* M2903(+) and M2903(−) isolates. Macrophages were infected with promastigotes of the two lineages of *L. braziliensis* M2903 and progression of infection was evaluated after 3 h (■), 6 h (□) and 24 h (▩). The results are expressed as infectivity indexes, calculated by multiplying the percentage of infected macrophages by the number of amastigotes present per cell.

Table 1
Infection of *Lutzomyia longipalpis* by *Leishmania braziliensis* M2903(+) and M2903(−). Results correspond to three independent infections of 50 females fed artificially and dissected after 7 days. T, total dissected females; P, total positive females.

	M2903(+)			M2903(−)		
	T	I	%	T	I	%
Experiment 1	11	0	0	15	1	6.7
Experiment 2	16	0	0	20	5	25
Experiment 3	17	0	0	20	5	25
Total	44	0	0	55	11	20

The inhibitory effect of methotrexate, that acts on DHFR-TS, was also studied; the two isolates presented a marked difference in sensitivity to this drug, with an EC_{50} of 8 mM for M2903(−) and 128 mM for M2903(+) (Fig. 3).

Reversion of methotrexate inhibition in the two L. braziliensis lineages

Lineages M2903(+) and M2903(−) were grown in the presence of 100 mM and 10nM methotrexate, respectively, the dose that inhibits 50% of cell proliferation, plus biopterin, folate and tetrahydrobiopterin in the concentrations used previously. In the case of M2903(+) the inhibition was reversed significantly (from 55% to 40%) in the presence of both tetrahydrobiopterin and folate. In the presence of biopterin alone the level of inhibition was identical to that obtained with only the addition of methotrexate. It is interesting to note that the presence of only tetrahydrobiopterin in the medium enhanced the inhibition, as well as the addition of biopterin and folate. In the case of M2903(−) the reversion of inhibition was significant in two instances: in presence of biopterin alone and in presence of both biopterin and folate, decreasing from 50% to 38% in the first case and from 50% to 8% in the second. Addition of tetrahydrobiopterin and tetrahydrobiopterin plus folate increased equally the inhibitory effect of methotrexate (Fig. 4).

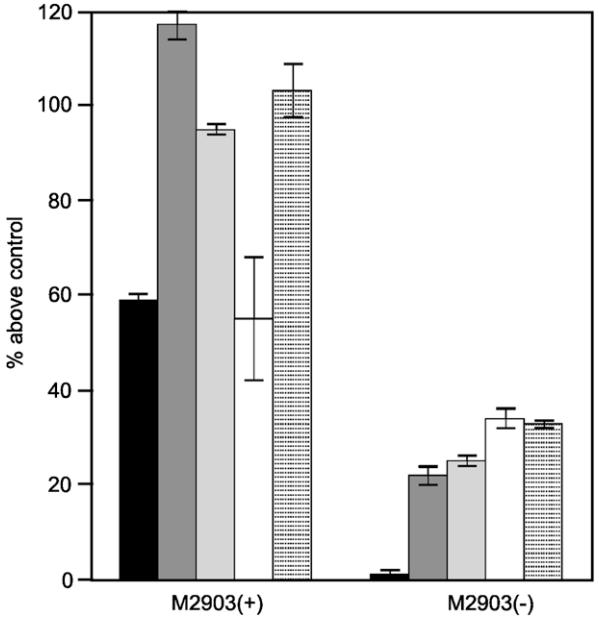

Fig. 2. Evaluation of cell growth of isolates M2903(+) and M2903(−) in the presence of metabolites of the biopterin salvage pathway. Parasites were grown in the presence of the indicated metabolites. Growth in the absence of metabolites was considered as the control. Results are show as % of growth above control. (■) 10 ng Fol; (▩) 15 ng Biopt; (▩) 100 ng BH_4; (□) 10 ng Fol + 15 ng Biopt; (▩) 10 ng Fol + 100 ng BH_4.

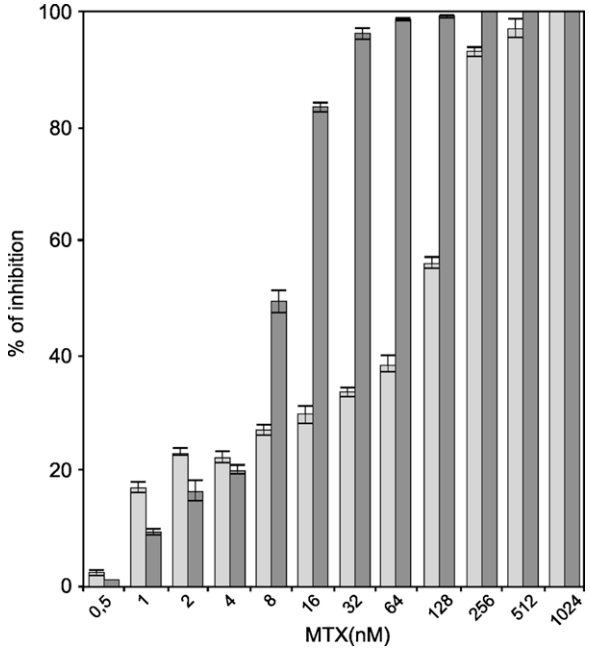

Fig. 3. Inhibitory effect of Methotrexate on the growth of *L. braziliensis*. (▩) M2903(+) and (▩) M2903(−) parasites were grown in the presence of increasing concentrations of MTX and counted.

Discussion

The underlying mechanisms and reasons for the appearance of spontaneous genomic amplifications in *Leishmania* are still unclear. The availability of two *L. braziliensis* lineages containing or not such amplification gave us the opportunity to ask some specific questions about their biological and metabolic specificities. Since previous work by others [11,14,17] and our group [12] identified a biopterin transporting protein in these amplicons, the presence of this gene was considered to be the possible force driving the appearance of LD1 type amplifications [13].

Treatment of leishmaniasis still relies on old drugs, and one of the approaches to search for new therapies has been to address the biochemical characterization of enzymes that participate in salvation pathways of these parasites. One of these is the folate biosynthetic pathway, since folates are essential coenzymes in

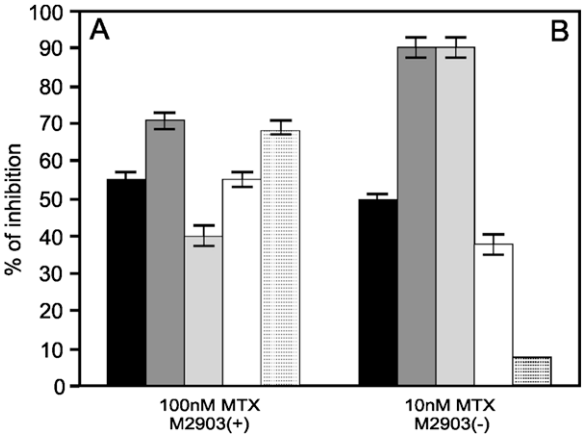

Fig. 4. Inhibition of cellular proliferation of *L. braziliensis* by methotrexate in the presence of folate, biopterin and tetrahydrobiopterin. Parasites were grown in the presence of methotrexate and the indicated metabolites. (■) Control + MTX; (▩) MTX + BH4 (100 ng/mL); (▩) MTX + BH4 (100 ng/mL) + Fol (10 ng/mL); (□) MTX + Biopt (15 ng/mL); (▩) MTX + Biopt (15 ng/mL) + Fol (10 ng/mL).

the synthesis of DNA and some amino acids, as well as in the initiation of protein synthesis. Folate sources are variable among different cell types; plants normally carry out a de novo synthesis while mammalian cells capture folate from exogenous sources [22]. *Plasmodium falciparum* utilizes the two sources [23]. *Toxoplasma gondii* not only to processes de novo synthesis, but also contains the genetic information for the production of folate transporters, capable of capturing the metabolite from the medium with high efficiency. *Leishmania* is completely dependent on exogenous sources of biopterin [24] and modulates the expression of genes coding for pteridin transporters. In this work we took advantage of the presence of multiple copies of BT1 transporter in an *L. braziliensis* lineage to investigate the influence of this transporter superexpression not only in the improved capture of pteridines but also in the virulence of this lineage, as compared to the normal lineage.

A marked difference was observed in the infective capability and virulence of the two lineages. The parasites containing the BT1 amplification were less successful in maintaining the infection in both the insect vector *L. longipalpis*, and macrophages 'in vitro', indicating that the differential capability to acquire biopterin influences infectivity and virulence. This observation could be related to the process of cellular differentiation in *Leishmania*, since the presence of metacyclic forms is essential for infectivity. In the macrophage infection experiments, late stationary parasites, that are considered to resemble metacyclic forms, were used [25]. Molecular events that participate in metacyclogenesis are not well understood. Experiments of Cunninghan et al. [26] in mice showed that a lowered production of tetrahydrobiopterin (BH_4) in *Leishmania* favors infection. These authors used an *L. major* strain knocked out for the PTR1 gene, and consequently with lowered levels of reduced folate and pteridine forms. In our experiments the *L. braziliensis* lineage without the amplification had a lower capacity to capture biopterin from the medium in relation to M2903(+), and, consequently, lower levels of BH_4. In macrophages, higher infectivity was seen in lineage M2903(−), even after 24 h of infection. A large difference in infectivity was detected in sandfly infection, being null in lineage M2903(+) and 20% in lineage M2903(−).

We observed that the more infective M2903(−) *Leishmania* lineage exposed more phosphatidyl serine (PS) on its surface (data not shown). PS exposure has been considered a signal for apoptotic cell death in unicellular eukaryotes [27], but only in *Leishmania amazonensis* it was related to recognition, signaling, and inhibition of microbicidal activity of the host cell [28]. These authors also show that most promastigote forms of *L. amazonensis* exposes PS in the outer leaflet of the plasma membrane, causing an exacerbation of infection and increasing the production of TGF-β1 and IL-10. Also, it was shown that in a virulent population of *Leishmania* the presence of apoptotic promastigotes was necessary for a successful infectious process [29]. This agrees with our results of a higher infectivity potential for the mammalian host.

In our experiments, the presence of the genomic amplification clearly favors the proliferation of one lineage in relation to the other. M2903(−) lineage growth rate in the presence of folate was similar to control, without any added metabolites. One possible explanation is that folate capture was small but sufficient, and carried out by normal BT1 levels, codified by 2 alleles on chromosome 35 and one on chromosome 27, or still by passive diffusion [30], considering that BT1 transports biopterin with high and folate with low affinity. It is interesting that, upon addition of folate together with biopterin or BH4, there was an approximately 30% increase of growth rate in relation to the increase originated by addition of folate alone, but smaller if compared to the addition of only biopterin and BH4. It seems that the capacity to capture folate or biopterin is more important than their internal metabolization, since the addition of the reduced form of the molecule (BH_4) had lower levels of growth. In lineage M2903(+) the amplification led to considerable increase in cell growth in the presence of folate (60%), biopterin (120%) and BH4 (95%) in relation to the control. The growth increase in the presence of biopterin was reverted when folate was also added in addition to biopterin, suggesting that the increased concentration of folate in the medium displaces the binding of biopterin to its transporter BT1. Considering that FT1 transports folate and MTX, the inhibition of M2903(+) growth by MTX was diminished by the presence of folate, probably by transporter competition.

Inhibition of DHFR-TS by MTX was not affected by increased capture of biopterin, since this enzyme is responsible for 90% of the reduction of biopterin into BH2 and BH4, only 10% of the process being under control of PTR1, which is much less sensitive to the action of MTX, in M2903(−) the presence of biopterin and mostly biopterin plus folate, reverted the inhibition by MTX. In this lineage the inhibitory effect of MTX was strengthened by the addition of the reduced form of biopterin, with or without folate. Considering there was 50% of inhibition, meaning there was half the basal level of biopterin being reduced, this strong inhibition could be due to excess metabolite.

The reoxidation of BH4 in the presence of molecular oxygen, in physiological pH, with the formation of superoxide ion has been shown [31], opposing the preexisting idea that BH4 itself could be a target for these deleterious radicals. The increased inhibition of growth by methotrexate in the presence of BH4, could be due to a similar mechanism generating oxidative stress inducing radicals. Therefore, besides the inhibition of conversion of folate to DHF, promoted by MTX, a concomitant production of hydrogen peroxide could represent an additional inhibitory stress factor. A stronger inhibitory effect was seen in M2903(−), that captures less biopterin, thus being more exposed to the effects of auto oxidation. The effect of folate, diminishing the inhibition, both in presence of biopterin and its reduced form BH4, is probably due to competition for a shared transporter.

Different cell types as fungi [32] and bacteria [33] make use of the apoptotic mechanism in order to favor their survival; probably habitats that lack essential nutrients for *Leishmania*, as the intestinal tract of sandflies, favor a similar behavior. Although the effective role of pteridines for the metabolism of *Leishmania* is somehow obscure, there are reports of increased metacyclogenesis upon pteridine depletion [26] as well as apoptotic signaling [34]. In this regard, our data suggest that a similar mechanism might be observed upon the infection of the vertebrate host with the *L. braziliensis* M2903(−) lineage.

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