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Reduced pathogenicity of fructose-1,6-bisphosphatase deficient *Leishmania donovani* and its use as an attenuated strain to induce protective immunogenicity

Savita Saini [a,b], Ayan Kumar Ghosh [b], Sushmita Das [c], Ruby Singh [b], Kumar Abhishek [b], Sudha Verma [b], Ajay Kumar [b], Abhishek Mandal [b], Bidyut Purkait [b], Kislay Kumar Sinha [a], Pradeep Das [a,b,*]

[a] Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Hajipur, Bihar, India
[b] Division of Molecular Biology, Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Indian Council of Medical Research, Patna, Bihar, India
[c] Department of Microbiology, All India Institute of Medical Sciences, Patna, Bihar, India

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ABSTRACT

Currently, there is no approved vaccine for visceral leishmaniasis (VL) caused by *L. donovani*. The ability to manipulate *Leishmania* genome by eliminating or introducing genes necessary for parasites' survival considered as the powerful strategy to generate the live attenuated vaccine. In the present study fructose-1,6-bisphosphatase (LdFBPase) gene deleted *L. donovani* (Δ*fbpase*) was generated using homologous gene replacement strategy. Though LdFBPase gene deletion (Δ*fbpase*) does not affect the growth of parasite in the promastigote form but axenic amastigotes display a marked reduction in their capacity to multiply *in vitro* inside macrophages and in vivo in Balb/c mice. Though Δ*fbpase L. donovani* parasite persisted in BALB/c mice up to 12 weeks but was unable to cause infection, we tested its ability to protect against a virulent *L. donovani* challenge**.** Notably, intraperitoneal immunisation with live Δ*fbpase* parasites displayed the reduction of parasites load in mice spleen and liver post challenge. Moreover, immunised BALB/c mice showed a reversal of T cell anergy and high levels of NO production that result in the killing of the parasite. A significant, correlation was found between parasite clearance and elevated IFNγ, IL12, and IFNγ/IL10 ratio compared to IL10 and TGFβ in immunised and challenged mice. Results suggested the generation of protective Th1 type immune response which induced significant parasite clearance at 12-week, as well as 16 weeks post, challenged immunised mice, signifying sustained immunity. Therefore, we propose that Δ*fbpase L. donovani* parasites can be a live attenuated vaccine candidate for VL and a good model to understand the correlatives of protection in visceral leishmaniasis.

1. Introduction

Leishmania causes a spectrum of diseases worldwide ranging from self-healing cutaneous to non-healing mucocutaneous and fatal form VL. There is currently no licensed vaccine against human leishmaniasis, although several vaccines are entered into clinical trials, most are still in early research and development [1]. The current drug treatments have the long half-life of the chemotherapeutics and is the reason for the emerging drug resistance among parasites [2,3].

Recovery from infection in natural cutaneous infection, cutaneous leishmaniazation [3–6], or visceral leishmaniasis confers immunity to re-infection. Killed parasites were though prophylactic but were inefficient in controlling disease [7,8]. In search of a vaccine, many biomolecules including recombinant proteins, poly-proteins [9], DNA [10] and combinations with or without adjuvant were tested in disease models or under clinical trials [1,11,12]. However, the notion that few live parasites inside host can generate lifelong protection suggests that successfully attenuated *Leishmania* could provide protection [3,13,14]. Live attenuated vaccine candidate provide a repertoire of antigen and stimulate immune response like a disease-causing agent, without pathogenesis and hence considered be more efficacious [15]. A vaccine against smallpox, measles, mumps, rubella and recent reports in plasmodium suggests that live attenuated pathogen is the gold standard for protection against specific pathogen [3,16,17].

Previously genes necessary for *Leishmania* amastigote survival and virulence were exploited for live attenuated vaccine candidate.

* Corresponding author at: Rajendra Memorial Research Institute of Medical Sciences, Agamkuan, Patna 800007, Bihar, India.
E-mail address: drpradeep.das@gmail.com (P. Das).

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It includes *L. major* dihydrofolate reductase-thymidylate synthase (dhfr-ts) [18], LPG2 [13], *L. mexicana* cysteine proteinase genes [19], biopterin transporter [20], *L. infantum* SIR2 [21], and *L. donovani* centrin ($LdCen^{-/-}$) genes [14]. dhfr-ts, lpg2, sir2 mutants due some inherent problems were excluded from future *Leishmania* vaccines. Most recently, $LdCen^{-/-}$ is reported to generate a protective immune response in mice, hamster, dog and also in patient's macrophages [14,22,23].

Safety, reproducibility and efficacy are of utmost importance characteristics of vaccine candidate [24]. Attenuated vaccines although open a novel approach to vaccine development, however, there are fears that the parasite may revert to a virulent form. Furthermore, targeted deletion of essential virulence genes can result in complete destruction of the parasite or mutants that only delay lesion development [25,26]. It is possible to refine the generation of *Leishmania* mutants, making them suitable for use as a live vaccine [27].

A Th1 type immunological response clears the parasites; it can trigger macrophages, which are the major cells to destroy *Leishmania* parasites. Immune response in VL is found to be mixed inflammatory and regulatory. Although VL initially was thought to be associated with a Th2 type immune response, most studies proposed that Th2 inclination in murine VL [28] and human VL [29] is not clear. IFNγ secretion is essential for parasite clearance in VL [30]. IL12 deficient mice were more susceptible to VL; they produce less IFNγ consequently displayed less inflammation during infection [31]. IL10 is strongly implicated to be associated with VL; it promotes parasites persistence by inhibiting macrophages activation [32–34]. IL10 inhibition leads to more rapid parasite clearance [35]. Blocking of the IL10 receptor leads to increased IFNγ production and enhanced expression of inducible nitric oxide synthase (iNOS) in infected tissue [36].

Sandfly injects *Leishmania* promastigotes into the host skin. They get ingested by various phagocytic cells but transforms into amastigotes only inside macrophage parasitophorous vacuole [37]. Nutrient-specific tropism of *Leishmania* amastigote might prevent it from invading other intracellular niches [38]. Particularly *Leishmania* amastigotes unable to colonize early endosome or non hydrolytic compartments which are lipid-rich but poor in aminoacids [39,40]. *Leishmania* is reported to utilise amino acids as an independent carbon source [39,40]. Based on available reports the glucose and other hexose metabolites including hexose amino sugars are essential for its survival. *Leishmania* amastigotes are likely to be found in hexose poor milieu and are dependent on de novo synthesis of sugar through gluconeogenesis. Gluconeogenesis deficient *Leishmania* was unable to replicate inside macrophage and in mice visceral organs [40–43]. *T. cruzi* amastigotes also reported being dependent on gluconeogenesis for the biosynthesis of glycoproteins and glycoinositol phospholipids [44]. To explore the essentiality of gluconeogenesis for the intracellular survival of *L. donovani* we studied the Fructose 1, 6-bisphosphatase (FBPase), the key enzyme catalysing the final committed step of this pathway. FBPase catalyses unidirectional hydrolysis of the fructose 1,6-bisphosphate (Fru-1,6-P2) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate [45,46].

In this study, *Ld*FBPase (Δ*fbpase*) null mutant parasite was generated. Δ*fbpase* amastigotes have reduced proliferation inside macrophage. *In-vivo* studies revealed reduced parasite load in Balb/c mice spleen and liver. Δ*fbpase L. donovani* were therefore tested for protection against WT parasites. Furthermore, a correlation between protective immune response (increased IFNγ, IL12 and decreased IL10 and TGFβ) was observed. It results in parasite clearance, which develops both cellular and humoral *Leishmania*-specific response. Thus, we suggested that Δ*fbpase* immunisation generates a protective immune response.

2. Material and methods

2.1. Statement of ethics

Five- to six-week-old female BALB/c mice from Central Drug Research Institute Luckhnow were used in the experiments. Eight-week-old mice were used in all the experiments. The Animal Ethical Committee of RMRIMS reviewed and approved the protocols for animal experiments. "The Guide for the Care and Use of Laboratory Animals," 8th edition were followed by the Institute for Laboratory Animal Research.

2.2. Cell culture of Leishmania donovani parasite

The parasites used in the study were *L. donovani* (MHOM/IN/83/AG83), which was originally isolated from an Indian VL patient and was maintained in golden hamsters. The amastigotes isolated from infected spleens of hamsters were converted into promastigotes at 24 °C in RPMI 1640 (Gibco) (pH 7.4) medium supplemented with 10% FBS (Gibco), 25 mM HEPES, 100 Uml^{-1} penicillin G sodium (Sigma–Aldrich, St Louis, MO, USA) and 100 mg ml^{-1} streptomycin sulfate (Sigma–Aldrich). The axenic cultures of promastigote stage of the parasite were maintained at 24 °C and subculture at every 72 h when cultures reached the confluence of 1–2 × 10^6 cells ml^{-1}.

2.3. Isolation of intracellular amastigotes

Intracellular amastigotes were isolated from infected mice PEC as described previously (26). Briefly, infected mice PEC were suspended in chilled PBS containing 1 mM EDTA and 11 mM Glucose. Then the suspension was passed five times through a 27-gauge needle. Cellular debris was removed by centrifugation (60*g* for 5 min at 4 °C), and the supernatant was passed through a 3 μm pore filter. Intracellular amastigotes were then recovered by centrifugation of the filtrate (800*g* for 10 min).

2.4. Semiquantitative PCR for LdFBPase gene expression at transcript level

Reverse transcription was performed using 5 μg of total RNA isolated from *L. donovani* from early log phase, stationary phase promastigotes and amastigotes (as described above). cDNA was generated using an anchored oligo-dT (Invitrogen) and used as a template to PCR amplify Ld*FBPase* (sense 5′-GCGAAGCTGTCCTTCAATGAGCAA-3′, antisense 5′-TATCGTGCTTGCCGTCGATCATCA-3′) ; and α-tubulin using initial denaturation at 94 °C for 5 min and 23 amplification cycles (30 s at 94 °C, 45 s at 56 °C and 1 min at 72 °C) and final extension for 5 min at 72 °C. The PCR products were run on a 2% agarose gel and stained with ethidium bromide (Sigma–Aldrich) before analysis. All the semi-quantitative PCR products were normalized to the α-tubulin semi-quantitative PCR product to ensure equal cDNA input.

2.5. Western blot analysis for LdFBPase gene expression at protein level

The *L. donovani* promastigotes (1 × 10^7 cells ml^{-1}) incubated under respective conditions were washed twice with 1X PBS, resuspended in sample buffer and proteins were separated by 10% SDS-PAGE electrophoresis and then transferred to PVDF membrane (Roche) which was followed by blocking with 3% BSA (Sigma–Aldrich). Immunoblots were probed with specific primary antibodies against LmFBPase (1:5000) followed by incubation with AP-conjugated goat anti-rabbit antibodies (1:5000) (Merck). Dilution of primary and secondary antibody was done with 1% BSA. The blot reacted with the primary and secondary antibody, was

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