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Recombinant *Leishmania tarentolae* expressing the A2 virulence gene as a novel candidate vaccine against visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis is the most severe form of leishmaniasis. To date, there is no effective vaccine against this disease. Many antigens have been examined so far as protein- or DNA-based vaccines, but none of them conferred complete long-term protection. The use of live attenuated vaccines has recently emerged as a promising vaccination strategy. In this study, we stably expressed the *Leishmania donovani* A2 antigen in *Leishmania tarentolae*, a non-pathogenic member of the genus *Leishmania*, and evaluated its protective efficacy as a live vaccine against *L. infantum* challenge. Our results show that a single intraperitoneal administration of the A2-recombinant *L. tarentolae* strain protects BALB/c mice against *L. infantum* challenge and that protective immunity is associated with high levels of IFN-γ production prior and after challenge. This is accompanied by reduced levels of IL-5 production after challenge, leading to a potent Th1 immune response. In contrast, intravenous injection elicited a Th2 type response, characterized by higher levels of IL-5 and high humoral immune response, resulting in a less efficient protection. All together, these results indicate the promise of A2-expressing *L. tarentolae* as a safe live vaccine against visceral leishmaniasis.

1. Introduction

Protozoan parasites of the genus *Leishmania* are causative agents of a broad spectrum of diseases, collectively known as leishmaniasis. The clinical symptoms of the disease range from asymptomatic self-healing cutaneous lesions (CL, caused by *Leishmania major*, *L. tropica*, and *L. mexicana* species) to mucocutaneous leishmaniasis (caused by *L. braziliensis*) and severe visceral (VL) infections (caused by *L. infantum*, *L. donovani*, and *L. chagasi*) [1,2]. Currently, over 12 million people in 88 countries are infected with this parasite, 350 million are at the risk of infection worldwide and 1.5–2 million new cases are reported every year [3–7]. VL is the most severe systemic disease among the three main categories of leishmaniasis and affects 500,000 people every year [6]. Moreover, VL has emerged as an opportunistic infection in HIV-1 infected patients in many parts of the world [8–10].

Currently, there is no effective vaccine against leishmaniasis and control of the disease is almost confined to chemotherapy. There are only a limited number of drugs available and each has its own disadvantages. Conventional drugs require long-term administration periods and often induce serious side-effects due to their toxicity [11,12]. In addition increasing incidence of drug-resistant strains has hampered the control of the disease by chemotherapy [13–17]. Therefore, much attention has been given to developing effective vaccines.

Although induction of lifelong protection against reinfection in recovered people demonstrates that a protective vaccine can be achieved, an effective vaccine against human leishmaniasis has yet to be discovered [18]. However, several vaccination strategies have been tried against experimental leishmaniasis, with particular emphasis on their efficacy against CL rather than VL [19,20]. First-generation anti-leishmanial vaccines, composed of killed parasites, were examined as an alternative but none has provided full protection thus far [18]. In addition, several antigens such as GP63 [21,22], GP46 [23], p36/LACK [24,25], CPB and CPA [26], LD1 [27], PSA-2 [28], TSA/LmSTI1 [29,30], PFR2 [31], HASPB1 [32], LeIF [33], LCR1 [34], and M2 [35] have been tested as recombinant proteins or DNA vaccines, but they have given only partial protection, and/or need some clinically unacceptable adjuvants to induce proper Th1 response in humans [36–38]. Also, it seems unlikely that a single

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antigen can elicit a fully protective immunity against a complex parasite like *Leishmania* [36]. On the other hand, there is increasing evidence that presence of a small number of live parasites is necessary to maintain durable immunity [39,40], and the only way to meet this requirement is by using attenuated live vaccines. Live vaccines can simulate the natural course of infection more closely, present a rich repertoire of antigens to the host immune system in the way that the wild type parasite does and persist for a longer time, leading to a more potent and durable immunity. Using live avirulent organisms as a vaccine has proven to be more efficacious than traditional subunit vaccines [41].

Attenuated strains using either long-term *in vitro* cultures [42] or selection for temperature sensitivity [43] or chemical mutagenesis [44], and parasite culture under drug pressure [45] are not easily applicable to human use, because there is always a risk of reversion of the organism to its virulent state. The advent of gene transfection technologies for *Leishmania* has made it possible to obtain attenuated organisms by targeting genes important for virulence or intracellular survival of the parasite, such as dihydrofolate reductase thymidylate synthase (DHFR-TS) [46], cysteine proteinase genes [47], HSP100 heat shock protein [48], SIR2 [49], LPG2 [50], biopterin transporter BT1 [51], and the trypanothione reductase [52]. Using intrinsically non-pathogenic species is also a promising approach, exemplified by BCG vaccine against *Mycobacterium tuberculosis* infection.

Among different species of *Leishmania*, *Leishmania tarentolae* is a lizard parasite which is non-pathogenic to humans. The parasite can differentiate into amastigotes, but is not able to persist long enough within mammalian macrophages [53,54]. Recent studies have shown that it can be used as a live vaccine against *L. donovani* due to its capability to efficiently target antigen-presenting cells, induce maturation of dendritic cells, and elicit a protective Th1 immune response [53]. Interestingly, a recombinant *L. tarentolae* expressing HIV-1 Gag protein has been used as a candidate HIV-1 vaccine and shown to induce strong cell-mediated immunity in mice and to decrease HIV-1 replication in an ex-vivo system [55], suggesting that this species can be used as a promising live-vectored vaccine against intracellular pathogens.

We have recently shown that among some important virulence factors, A2 is not present in *L. tarentolae* [56]. A2 was first identified in *L. donovani* as a gene family that is expressed only in the amastigote stage [57]. A2 proteins are comprised mainly of a 10-amino acid repeated sequence and range in size from 45 to 110 kDa, depending on the number of repeats [58]. A2-specific antibodies have been detected in 90% of sera samples from VL patients, which confirms that A2 is expressed in the human host [59]. In addition, a significant protection against *L. donovani* and *L. amazonensis* infections associated with both humoral and cellular immune responses has been obtained when using A2 as a recombinant protein or a DNA vaccine in BALB/c mice, and more recently in dogs [60–65].

In this study, we engineered a recombinant *L. tarentolae* expressing the *L. donovani*-specific A2 protein and used it as a live vaccine against *L. infantum* infection in BALB/c mice. We provide evidence that intravenous (i.v.) and to a larger extent intraperitoneal (i.p.) immunization with the recombinant *L. tarentolae*-A2 strain elicited favorable immune responses and significant levels of protective immunity against *L. infantum* infectious challenge.

2. Materials and methods

2.1. DNA constructs

The *A2* gene (with 3′UTR sequences) was amplified from pKSNEOA2-1 vector [58] (kindly provided by Dr. Greg Matlashewski, Microbiology and Immunology Department, McGill University, Montreal, Quebec, Canada) by hot-start PCR (HotStar kit, Qiagen) using the following primers: (forward, 5′-TTGAAGCTTACCGAGCACAATGAAGATCC-3′); and (reverse, 5′-AACAAGCTTAGCAGAGGAAGTCAGCAAGG-3′) including *Hin*dIII restriction sites indicated as underlined. For amplification, DNA was denatured at 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 90 s, and a cycle of 72 °C for 10 min. The amplified fragment was cloned into the pDrive cloning vector (Qiagen). After sequence confirmation, the A2 fragment was subcloned into the *Hin*dIII site of vector pNEO-GFP [66] upstream of the *GFP* gene to generate pNEO-GFP-A2, and the correct insert orientation was confirmed by restriction analysis.

2.2. Parasite growth and transfections

The *L. tarentolae* Tar II (ATCC 30267) parasites were grown at pH 7.2 and 26 °C in M199 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 40 mM HEPES, 0.1 mM adenosine, 5 μg/ml hemin and 50 μg/ml gentamicin. For transfection, 4×10^7 log-phase parasites were washed and re-suspended in 300 μl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose; pH 7.5) containing 15 μg of plasmid DNA, stored on ice for 10 min, and electroporated using Bio-Rad Gene Pulser Ecell at 450 V and 500 μF as described previously [67]. Transfectants were selected with G418 (Sigma).

2.3. RNA extraction and reverse-transcription PCR

For *L. tarentolae*, RNA samples were extracted from both promastigote and amastigote-like forms of the parasite. *L. tarentolae* amastigote-like forms were prepared by culturing the parasites at pH 4.5 and 37°C for 10 h. In the case of *L. infantum*, amastigotes were obtained by infecting mouse peritoneal macrophages with stationary-phase parasites. Briefly, peritoneal exudate cells from naïve BALB/c mice were used as a source of resident macrophages (MQ). The peritoneal cavity was washed with 5 ml cold RPMI 1640 (Sigma) media, and then the media containing peritoneal macrophages was recovered. Cells were cultured in RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, 50 nM 2-mercaptoethanol, 10 mM HEPES and 40 μg/ml gentamicin at a density of 10^6 cells/well, and incubated at 37 °C in 5% CO_2 for 24 h. In order to infect MQ, the stationary-phase *L. infantum* promastigotes were added at the ratio of 10:1 parasites versus macrophages. The cultures were washed after 4 h to exclude free parasites and then incubated for 5 h. Infected attached MQs were collected using cold PBS. RNA was extracted using RNeasy kit (Qiagen) and treated with RNase-free DNase for 30 min at 37 °C to eliminate any remaining DNA. cDNA synthesis was performed using the Qiagen Omniscript RT Kit from 1 μg of RNA. To detect the A2 cDNA, two primers were designed to amplify a 154-bp region within the A2 3′-UTR starting at the proximity of A2 stop codon (AUF: 5′-GCAAGAGCAACAGCAGAGC-3′; AUR: 5′-CTTCCAACCTTGCGACTTTC-3′), and a hot-start PCR (Qiagen HotStar Kit) was performed at 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and a cycle of 72 °C for 10 min.

2.4. Northern blot hybridization

Northern blot analysis was performed with the DIG System (Roche Applied Science) according to instructions of the manufacturer with minor modifications. For each sample, 3 μg of total RNA was denatured, blotted onto Hybond + membrane (Roche), and UV cross-linked to the membrane with the intensity of 0.12 J/cm^2. Using the "PCR DIG Probe Synthesis Kit" (Roche Applied Science), a DIG-labeled probe was prepared by PCR amplification of a 1.7 kb