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A prime-boost immunization with Tc52 N-terminal domain DNA and the recombinant protein expressed in *Pichia pastoris* protects against *Trypanosoma cruzi* infection

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ABSTRACT

We have previously reported that the N-terminal domain of the antigen Tc52 (NTc52) is the section of the protein that confers the strongest protection against *Trypanosoma cruzi* infection. To improve vaccine efficacy, we conducted here a prime-boost strategy (NTc52PB) by inoculating two doses of pcDNA3.1 encoding the NTc52 DNA carried by attenuated *Salmonella* (SNTc52), followed by two doses of recombinant NTc52 expressed in *Picchia pastoris* plus ODN-CpG as adjuvant. This strategy was comparatively analyzed with the following protocols: (1) two doses of NTc52 + ODN-CpG by intranasal route followed by two doses of NTc52 + ODN-CpG by intradermal route (NTc52CpG); (2) four doses of SNTc52; and (3) a control group with four doses of *Salmonella* carrying the empty plasmid. All immunized groups developed a predominant Th1 cellular immune response but with important differences in antibody development and protection against infection. Thus, immunization with just SNTc52 induces a strong specific cellular response, a specific systemic antibody response that is weak yet functional (considering lysis of trypomastigotes and inhibition of cell invasion), and IgA mucosal immunity, protecting in both the acute and chronic stages of infection. The group that received only recombinant protein (NTc52CpG) developed a strong antibody immune response but weaker cellular immunity than the other groups, and the protection against infection was clear in the acute phase of infection but not in chronicity. The prime-boost strategy, which combines DNA and protein vaccine and both mucosal and systemic immunizations routes, was the best assayed protocol, inducing strong cellular and humoral responses as well as specific mucosal IgA, thus conferring better protection in the acute and chronic stages of infection.

1. Introduction

Trypanosoma cruzi is a protozoan intracellular parasite and the etiological agent of Chagas disease. The transmission modes for this parasite are diverse, namely vectorial, through blood transfusion and organ transplantation, vertically from mother to infant, and by oral route. The infection has an initial acute stage followed by a chronic stage, which can be symptomatic or not. Up to 30% of chronically infected individuals develop cardiac alterations whereas 10% of them develop digestive, neurological or mixed alterations [1]. Treatment is based on one of two drugs: nifurtimox and benznidazole. Both are effective in the acute stage of infection, losing effectiveness in the advanced phase. Furthermore, severe side effects are associated with the treatment. Currently, the number of worldwide infected individuals is estimated in 8-10 million. The disease affects not only people from endemic areas in South and Central America, but also from many countries of Western Europe and North America [1,2]. Efforts are focused not only in transmission control, but also in the development of more efficient and less toxic drugs as well as prophylactic and therapeutic vaccines.

In the field of vaccine development against *Trypanosoma cruzi*, several antigens such as cruzipain (Cz), amastigote surface protein 2 (ASP-2), trans-sialidase (TS), gp82 and paraflagellar rod proteins

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(PFR) have been tested among others, [reviewed in 3,4]. Recent strategies included DNA delivery systems comprising attenuated viruses and bacteria [5–8]. This kind of immunization induces a significant cellular immune response and the cytotoxic T cell activation required to control infection [9,10]. However, it has been shown that even when the cellular immune response is crucial for protecting against infection, antibodies are also required [11–13].

Tc52 is a *T. cruzi* protein having glutathione transferase activity [14], whose sequence is highly conserved among strains [15]. It is crucial for parasite survival since the knockout of both alleles is lethal [16]. Tc52 has many immunomodulatory properties: (1) it inhibits splenocyte proliferation induced by mitogen [17,18]; (2) it binds to dendritic cells (DC) and macrophage surface [19]; (3) it increases the expression of inducible nitric oxide synthase (iNOS) and nitric oxide production by macrophages in the presence of IFN-γ [20], among other properties. All these characteristics make Tc52 a promising vaccine candidate.

Tc52 has two domains: the N-terminal domain (NTc52) that has a molecular weight of 26 kDa and contains the enzyme active site, and a 25 kDa C-terminal (CTc52) domain, whose function is still unknown but could be responsible for some immunomodulatory activities [18]. The ability of recombinant Tc52 or its naked DNA to protect against *T. cruzi* infection yielded promising results [19,21]. More recently, we evaluated the ability of a *Salmonella* DNA delivery system encoding Tc52 and its N-and C-terminal domains to protect against *T. cruzi*. NTc52 conferred greater protection than CTc52 or full length Tc52 in the acute and chronic stages of infection [22]. The main goal of the present work was to improve the immune protection elicited by *Salmonella* as DNA delivery system of NTc52 using a prime-boost strategy.

Pichia pastoris is a eukaryotic organism that is easy to grow, allowing the production of recombinant proteins in large quantities. In addition, using suitable vectors, the protein of interest could be addressed either to the intracellular compartment or the secretion system. In the last few years, many recombinant proteins, including *T. cruzi* proteins, have been produced using *P. pastoris* as expression system [23,24]. We have previously expressed NTc52 as a recombinant protein in *E. coli* [22]. In this work we have chosen *P. pastoris* as expression system to improve soluble protein production.

2. Materials and methods

2.1. Parasites

T. cruzi epimastigotes (RA strain) were grown in LIT medium [22]. *T. cruzi* bloodstream RA trypomastigotes and the recombinant Tulahuen strain expressing β-galactosidase (Tul-β-Gal) [25] were isolated from infected mice.

2.2. Tc52 and NTc52 cloning and expression in Pichia pastoris

Tc52 and its N-terminal domain (residues 1–223) were cloned into pPICzα-A plasmids. For that purpose, genomic DNA was extracted from RA epimastigotes. The sequences were amplified by PCR using the following sets of primers: for full-length Tc52, the forward primer 5′-CGACTGGAATTCATGAAGGCTTTGAAACTTTTTAAAGA-3′ containing an *Eco*RI restriction site (underlined) and the reverse primer 5′-ACTAGCGCGGCCGCTCAGTGATGGTGATGGTGATGAGACGATGGAC-GCAA-3′ with a *Not*I restriction site and a sequence encoding a His_6 tag (both underlined). For the N-term domain, the Tc52 forward primer and the reverse primer 5′-ACTAGCGCGGCCGCTCAGTGATGGTGATGGTGATGCAATGACCAT-GTGACGTGC-3′ also with a *Not*I restriction site and a His_6 tag. The PCR products of 1,382 (Tc52) and 722 bp (NTc52) were digested with *Eco*RI and *Not*I and cloned in vector pPICzα-A (Invitrogen). Cloning was performed in *E. coli* DH5α, selecting positive clones by Zeocin resistance. Both pPICzα-Tc52 and pPICzα-NTc52 constructions were purified from the selected clones and sequenced.

Plasmids pPICzα-Tc52 and pPICzα-NTc52 were linearized with *Sac*I restriction enzyme, purified and used to transform 10^{10} electrocompetent *P. pastoris* KM71 or GS115 cells: pulsed 1.5 kV, 25 μF, 200 Ω, and then incubated for 2 h in cold 1 M sorbitol [26]. Recombinant yeast selection was performed in YPDS-Zeocin agar plates. Different Zeocin concentrations in the range 100-1000 mg/ml were tested, and clones with resistance to higher antibiotic concentrations were selected. The insertion of the DNA fragment was checked by colony PCR in selected Zeocin-resistant colonies [27]. PCR positive clones were cultured in minimal methanol histidine plates. Cultures were conducted at 28 °C for 4 days, daily adding methanol. Colony blotting was then assayed as described [26] using a mouse-specific anti-Tc52 or anti-NTc52 [22]. The yeasts colonies with higher expression levels were amplified as recommended (Invitrogen). Cultures (100 ml) were grown until $DO_{600\,nm}$ = 0.6–0.8. For protein expression, cells were centrifuged for 10 min at 2000 × *g* and suspended in 150 ml of BMMH and cultured for 5 days Methanol was added daily to 0.5% final concentration. Based on previous reports [26,28], other methanol concentrations (1%, 1.5%, 2% and 2.5%) were evaluated to enhance yield.

Recombinant Tc52 and NTc52 were purified under native conditions. *P. pastoris* induced-cultures were centrifuged at 4 °C, 20,000 × *g* for 30 min. Supernatants were concentrated 10 times by centrifugal ultrafiltration (Amicon, Millipore). Prior to purification, concentrated supernatants were dialyzed against PBS supplemented with 10 mM imidazole. Purifications of both proteins were performed in a nitrilotriacetic acid (NTA) column, at 4 °C, with purification buffer by imidazole gradient: 25 mM, pH 8.0, for washes, and 250 mM, pH 7.5, for elution. Purified proteins were then dialyzed against phosphate-buffered saline (PBS) and analyzed by SDS-PAGE and immunoblotting to verify purity and identity [22].

2.3. Cloning and expression of the N-terminal domain in a eukaryotic expression system

Cloning and expression of NTc52 in pcDNA3.1 eukaryotic plasmids and transformation of attenuated *Salmonella enterica* serovar Typhimurium *aroA* SL7207 were previously described [22].

2.4. Immunizations and challenge

Four groups (10 animals/group) of 6–8-week-old inbred female C3H/HeN mice were immunized four times every 10 days as follows: GI (control group): 10^9 CFU of *Salmonella* carrying the pcDNA3.1 vector (Sempty) by oral route; GII: 10^9 CFU of *Salmonella* harboring construct pcDNA3.1-NTc52 (SNTc52); GIII: a prime-boost vaccination protocol (PB) with 2 oral doses of SNTc52 and 2 doses of rNTc52 + ODN-CpG 1826 as adjuvant, by intradermal route (NTc52PB); GIV: rNTc52 + ODN-CpG 1826: two doses by intranasal route, and two by intradermal route (NTc52CpG). Intradermal and intranasal immunizations were performed with 10 μg of rNTc52 and 20 μg of ODN-CpG. For oral immunizations, attenuated *Salmonella* carrying the constructions (empty pcDNA3.1 or pcDNA3.1-NTc52) were grown in Brain Heart Infusion (BHI) medium supplemented with 100 μg/ml ampicillin, at 37 °C 70 rpm. Cultures reaching OD_{600} = 0.6 were centrifuged and suspended in 2.65% $NaHCO_3$ buffer, supplemented with 1.65% ascorbic acid and 0.2% lactose. Mice were deprived of water 2 h before immunization with 20 μl of the suspension (10^9 CFU). The number of CFU

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