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Identification of novel vaccine candidates for Chagas' disease by immunization with sequential fractions of a trypomastigote cDNA expression library

Valeria Tekiel[a,*,1], Catalina D. Alba-Soto[b,1], Stella M. González Cappa[b], Miriam Postan[c], Daniel O. Sánchez[a]

[a] Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín, CONICET, Buenos Aires, Argentina
[b] Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
[c] Instituto Nacional de Parasitología Dr. M. Fatala Chaben, ANLIS-Malbrán, Buenos Aires, Argentina

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ABSTRACT

The protozoan *Trypanosoma cruzi* is the etiological agent of Chagas' disease, a major chronic infection in Latin America. Currently, there are neither effective drugs nor vaccines for the treatment or prevention of the disease. Several *T. cruzi* surface antigens are being tested as vaccines but none of them proved to be completely protective, probably because they represent only a limited repertoire of all the possible *T. cruzi* target molecules. Taking into account that the trypomastigote stage of the parasite must express genes that allow the parasite to disseminate into the tissues and invade cells, we reasoned that genes preferentially expressed in trypomastigotes represent potential targets for immunization. Here we screened an epimastigote-subtracted trypomastigote cDNA expression library by genetic immunization, in order to find new vaccine candidates for Chagas' disease. After two rounds of immunization and challenge with trypomastigotes, this approach led to the identification of a pool of 28 gene fragments that improved *in vivo* protection. Sequence analysis of these putative candidates revealed that 19 out of 28 (67.85%) of the genes were hypothetical proteins or unannotated *T. cruzi* open reading frames, which certainly would not have been identified by other methods of vaccine discovery.

1. Introduction

Chagas' disease currently affects ~18 million people and more than 100 million people in Latin America are potentially exposed to its causative agent, the protozoan *Trypanosoma cruzi*. Available treatments are limited to chemotherapies that exhibit high toxicity and display poor efficacy at the chronic stage of the disease [1]. Up to date, there is no effective vaccine against *T. cruzi* [2].

Several parasite antigens have been tested for their utility in controlling *T. cruzi* infection and/or disease (cruzipain, *trans*-sialidase (TS), amastigote surface protein-2, trypomastigote surface antigen-1, or mucins, among others) [3–10]. These antigens were chosen to be tested as vaccines mainly for their localization on the parasite surface and the induction of strong cellular and humoral responses against them during natural infection. However, they represent a limited repertoire of all the possible *T. cruzi* target molecules. Moreover, most of these antigens belong to large gene families whose size and variability are one of the parasite's mechanisms to evade the host immune system [11]. Accordingly, the magnitude of the immune response elicited solely by these antigens might be insufficient to control the infection. Previous experiences on vaccine discovery to others pathogenes that must be targeted by multiple immune responses as *Plasmodium* sp. indicate that protective immune responses could be better achieved by multivalent vaccines able to emulating the complexity of immune responses attained during natural infection, but given in formulation capable of increasing the immunogenicity of antigens [12,13]. Thus, additional target molecules remain to be identified for vaccine development against Chagas disease.

T. cruzi has multiple developmental stages cycling between a reduviid insect vector and a mammalian host. The replicative epimastigote and the non-replicative metacyclic trypomastigote are the parasite forms present in the vector; the latter is the infective stage when *T. cruzi* is transmitted in endemic areas. Focusing on the mammalian host, the circulating trypomastigote stage is a hallmark of the acute infection, when found in high numbers in the blood. The trypomastigote is responsible for the invasion of nucleate cells, where the parasite differentiates to amastigote, the stage that multiplies in the cytoplasm. Then, amastigotes differentiate to trypomastigotes, the infected host cell bursts, the parasites reach

* Corresponding author at: Instituto de Investigaciones Biotecnológicas, IIB-UNSAM, Av. Gral Paz 5445, Parque Tecnológico Miguelete, Edificios 19 and 24, B1650KNA, San Martín, Buenos Aires, Argentina. Tel.: +54 11 4580 7257; fax: +54 11 4580 7257.
E-mail addresses: valet@iib.unsam.edu.ar, vtekiel@gmail.com (V. Tekiel).
[1] These two authors contributed equally to this work.

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the bloodstream and disseminate the infection to diversal host tissues. Trypomastigotes are also the infective stage in congenital and transfusional modes of transmission, situations that take place even outside endemic areas. Therefore, this parasitic stage represents a promising target for the control of Chagas' disease.

Recently, with the purpose of generating information about genes preferentially expressed in trypomastigotes but not in epimastigotes, we have constructed a trypomastigote cDNA library subtracted with epimastigote cDNA (library TcT-E). We identified ~180 new *T. cruzi* expressed sequence tags, by means of automatic sequencing and database mining of a small portion of the TcT-E library, and confirmed experimentally that those clones were preferentially represented in the trypomastigote stage (Tekiel et al., unpublished results; http://genoma.unsam.edu.ar/projects/tct-e/tct-e.c.html). We consider that the product of some of these genes could be new potential vaccine candidates for Chagas' disease, but the lack of knowledge of their function makes it impossible to rationally select those suitable for vaccine trials.

The expression library immunization (ELI) is an attractive strategy for the identification of novel vaccine candidates, and, unlike traditional and *in silico*-based approaches, it makes no assumptions and requires no prior knowledge of antigenic targets [14]. Basically, expression libraries are used for genetic immunization and, after challenge with the pathogen, the host's immune system selects the immunoprotective clones. The method implies the fractioning of the library into smaller sub-libraries or pools, that are sequentially screened *in vivo* until a group or even single protective genes are identified [15–21]. ELI has the potential for screening the complete genome of a pathogen but, when the complexity of genomes is high, the coding density is low or when stage-specific vaccines are required – as is the case of *T. cruzi* – a modification of ELI starting from cDNA is more convenient [22].

We therefore used the subtractive trypomastigote cDNA library as starting point to allow the immune system of the murine host model of Chagas' disease to select protective antigens. After two rounds of genetic immunization and challenge, this approach led to the identification of novel – otherwise unpredictable – *T. cruzi* vaccine candidates.

2. Materials and methods

2.1. Animals, parasites and antigens

C3H/HeN^{k} mice were bred and housed in our animal facility (Department of Microbiology, Parasitology and Immunology, School of Medicine, University of Buenos Aires). All procedures requiring animals were performed in agreement with institutional guidelines.

The RA strain of *T. cruzi* was kept *in vivo* by weekly intraperitoneal passages of 10^5 bloodstream forms through mice [23].

For parasite lysate preparation, trypomastigotes from the CL Brener strain were obtained from supernatants of *in vitro* infected Vero cells. Cultured trypomastigotes (with less than 3% amastigote forms) were harvested, washed in PBS and subjected to five freeze–thawing cycles and sonication (10 cycles of 30 s at 40 Hz on ice). Protein concentration was determined by the Bradford method and the lysate was stored at −80 °C until use.

2.2. Epimastigote-subtracted trypomastigote cDNA library (subtractive TcT-E library) construction

The subtractive TcT-E library was constructed by using the PCR-Select cDNA Subtraction kit following the selective subtractive hybridization protocol provided by the manufacturers (CLONTECH, USA) [24]. First-strand cDNA synthesis was performed with 2 μg of polyA+ of each *T. cruzi* stage (trypomastigote and epimastigote, CL Brener strain), oligo dT primer with a 5′ RsaI site and Superscript II reverse transcriptase (Gibco-BRL, USA). Second-strand cDNA synthesis was performed with T4 DNA polymerase. After RsaI digestion of double-stranded cDNA, two different sets of adaptors were ligated to the tester cDNA (trypomastigotes) but not to the driver cDNA (epimastigotes). Two rounds of subtractive hybridization in the presence of an excess of epimastigote cDNA were performed, leading to the enrichment of differentially expressed sequences in the trypomastigote cDNA population. This subtracted sample was the template for further suppression PCR amplification performed with adaptor-specific primers. The subtraction efficiency was verified by monitoring the PCR amplification of *T. cruzi* histone 2A transcript in subtracted and unsubtracted samples (H2_3′: tcttggacgccttcttcgct; H2_5′: gtgatgccgagcctgaacaa) and by reverse Northern blot. PCR products enriched for trypomastigote differentially expressed sequences – higher than 100 bp – were cloned into the pGEM-T Easy vector (Promega, USA).

2.3. Construction of the expression library ELI_TcT-E

2.3.1. Vector construction

Eukaryotic expression vectors pCI30, pCI31 and pCI32 constructed as described by Moore et al. (2002) were a kind gift of Dr. D. Comerci and Dr. J.E. Ugalde [25]. For the present project, the NotI site of pCI30-32 was removed and a new NotI site added next to the BamHI site. Each of the three new vectors – pCI-Not30, pCI-Not31, pCI-Not32 – has the NotI cloning site in a different reading frame. This site was used for the subcloning of inserts released from the subtractive TcT-E library using the same restriction enzyme.

2.3.2. Preparation of inserts

Plasmid DNA from the original TcT-E library, cloned in pGEM-T Easy vector, was purified by the alkaline lysis method and digested with NotI. Released inserts were gel-purified according to their size (<400 bp, ~500 bp, 500–700 bp and >700 bp) using QiaexII (QIAGEN Inc., GmbH, Germany) and separately cloned into the pCI-Not30, pCI-Not31 and pCI-Not32 vectors. Each of the 12 ligations (pCI-Not30–32 ligated with each of the four different size-range inserts) was used to transform *E. coli* DH5α by standard procedures [26].

2.4. Selection of polyHis-positive clones

Colonies lifts were taken from the transformation plates onto nitrocellulose filters. To induce the expression of polyHis recombinant peptides, the filters were placed onto LB agar containing 2 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and incubated for 4 h. Then, filters were treated with denaturing and neutralization solutions, blocked with 3% BSA in TBS and incubated with an anti-polyHis monoclonal antibody (Penta-His Antibody, 1/1000 dilution, Qiagen). The detection of expressing clones was carried out with a secondary antibody coupled to peroxidase and developed with a staining solution containing 4-chloro-1-naphthol and H_2O_2. Filters were aligned with the original transformation plates and the polyHis-positive clones representing the ELI_TcT-E library were picked into 384 plates in *Hogness Modified Freezing Medium* (*HMFM*). After overnight growth at 37 °C, replicates were made for long-term storage at −80 °C.

2.5. Expression library arrangement and deconvultion

A first master plate containing the productive clones for each cloning vector (pCI-Not30, 31 and 32) was made in 384-well plates to further easily reorder the clones. From these initial master plates, we subsequently picked the clones to construct the different sub-libraries used for the immunization assays. The