Comparative evaluation of therapeutic DNA vaccines against *Trypanosoma cruzi* in mice

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**Abstract**

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a major public health problem in most of Latin America. A key priority is the development of new treatments, due to the poor efficacy of current ones. We report here the comparative evaluation of therapeutic DNA vaccines encoding various *T. cruzi* antigens. ICR mice infected with 500 parasites intraperitoneally were treated at 5 and 12 days postinfection with 20 mg of plasmid DNA encoding *T. cruzi* antigens TSA-1, TS, ASP-2-like, Tc52 or Tc24. Treatment with plasmid encoding TS and/or ASP-2-like antigens had no significant effect on parasitemia or survival. Treatment with Tc52 DNA significantly reduced parasitemia, as well as cardiac parasite burden, and improved survival, although myocarditis was not significantly affected. Finally, treatment with plasmids encoding Tc24 and TSA-1 induced the most complete control of disease as evidenced by significant reductions in parasitemia, mortality, myocarditis and heart parasite burden. These data demonstrate that therapeutic vaccine efficacy is dependent on the antigen and suggest that DNA vaccines encoding Tc24, TSA-1, and Tc52 represent the best candidates for further studies of a therapeutic vaccine against Chagas disease.

**Introduction**

Chagas disease, or American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi*, and it affects an estimated 16–18 million persons, with close to 100 million people at risk of infection in the Americas. About 30–40% of infected patients develop chronic chagasic cardiomyopathy, which represents a dramatic burden in public health (WHO, 2002).

There has been extensive debate on the mechanisms involved in this pathology (Kierszenbaum, 1999; Tarleton, 2001; Girones & Fresno, 2003). Part of the susceptibility of patients to develop chronic chagasic cardiomyopathy has been associated with specific major histocompatibility complex (MHC) haplotypes (Colorado et al., 2000; Cruz-Robles et al., 2004). In addition, some studies suggested that tissue damage was associated with the presence and replication of intracellular amastigotes, and others proposed that autoimmunity induced by parasite antigens mimicking host proteins was responsible for tissue damage (Kierszenbaum, 1999; Tarleton, 2001; Girones & Fresno, 2003). It is now accepted that the presence of parasites in cardiac tissue is necessary to initiate and maintain the inflammatory response, and that therapeutic treatments or vaccines aimed at eliminating *T. cruzi* would limit or prevent the progression towards chronic chagasic cardiomyopathy (Bhatia & Garg, 2005).

Current chemotherapy relies on nitrofurans (Nifurtimox), or nitroimidazoles (Benznidazole). However, the usefulness of these drugs is limited by their reduced efficacy (mostly during early stages of the infection), serious side effects, and the emergence of drug-resistant strains of parasites (Castro et al., 2006). Efforts are thus urgently needed to identify new drug candidates. A number of compounds have been evaluated in preclinical models for the chemotherapy of *T. cruzi* infection and are providing some variable but encouraging results (Buckner et al., 2003; Urbina & Docampo, 2003; Steverding & Tyler, 2005). Nonetheless, a major limitation of chemotherapy is that most treatments require very long administration regimens to be effective.

Therapeutic vaccines, aimed at reinforcing the immune response of an infected host, represent a novel and attractive
alternative to chemotherapy against chronic diseases (Autrann et al., 2004). The major advantages of this strategy are that it relies on short treatment regimens (Lai et al., 1996; Boyer et al., 1997; Lowrie et al., 1999; Lodmell & Ewalt, 2001; Bahoul et al., 2003), and the induction of multiple effector mechanisms against the pathogen may have high efficacy and lower the possibilities of resistance. In particular, we provided proof-of-concept data demonstrating that two doses of therapeutic DNA vaccines encoding parasite antigens TSA-1 or Tc24 could reduce disease severity in mice infected with an otherwise lethal dose of \textit{T. cruzi} (Dumonteil et al., 2004). Importantly, this treatment is effective at reducing disease development when administered at various times during both the acute and the chronic phase of the infection, although efficacy decreases when treatment is delayed (Dumonteil et al., 2004). The efficacy of such therapeutic DNA vaccines seems to rely, at least in part, on the induction of parasite-specific interferon gamma (IFN-\(\gamma\))-producing CD\(4^+\) and CD\(8^+\) T cells (Zapata-Estrella et al., 2006), confirming that stimulation of the immune response by the vaccine limits disease progression rather than exacerbates tissue inflammation and damage. This is also in agreement with several studies showing that a T helper (Th) 1 type immune response is required for clearing the parasite from infected mice (Silva et al., 1995; Miller et al., 1996; Santori et al., 1996; Paiva et al., 1999; Hoft & Eickhoff, 2002) and that less severe disease is observed in patients with higher frequency of IFN-\(\gamma\)-producing T cells (Laucella et al., 2004). Various vaccine studies have further established that to be protective against \textit{T. cruzi}, antigens need to contain both MHC I- and II epitopes, and lead to a full activation of the immune response, including helper CD\(4^+\) and cytotoxic CD\(8^+\) cells, as well as lytic antibodies (Bhatia & Garg, 2005).

Nonetheless, most of these studies have focused on antigens from the blood-form trypomastigote stage of the parasite, and antigens from intracellular amastigotes have been much less explored (Low et al., 1998; Garg & Tarleton, 2002; Boscardin et al., 2003). There is indeed a good rationale for designing vaccines aimed at either stages of the parasite, and it would be expected that vaccines targeting the circulating or the intracellular stages would be equally effective at controlling disease progression. Thus, it remains unclear how the nature of the antigens may affect vaccine efficacy and which may be the most promising for the detailed study of the immune mechanisms underlying their therapeutic efficacy.

To address this issue and identify new therapeutic vaccine candidates, we report here the comparative evaluation of the efficacy of vaccines encoding several \textit{T. cruzi} antigens, using a convenient acute phase model. We selected ASP-2-like clone 9, an antigen whose mRNA is observed in all developmental stages of the parasite, but the full length protein is only detected in amastigotes (Boscardin et al., 2003).

Similarly, \textit{T. cruzi} 52 kDa antigen (Tc52) is mostly expressed in amastigotes and epimastigotes, and downregulated in trypomastigotes, and it is considered a virulence factor (Ouaissi et al., 1995). We also investigated two trypomastigote-specific antigens from the trans-sialidase family, the trans-sialidase (TS) (Abuin et al., 1999) and the trypomastigote-surface antigen 1 (TSA-1) (Fouts et al., 1991), although some members of this family are also expressed in amastigotes, and may provide some cross-reactions. Finally, we included \textit{T. cruzi} 24 kDa antigen (Tc24), which is present in all developmental stages of the parasite (Ouaissi et al., 1992; Taibi et al., 1993). Well-defined MHC I and II epitopes have been identified in most of these antigens and they are able to induce a strong and complete immune response in mice (Taibi et al., 1993; Wrightsman et al., 1994; Wizel et al., 1998; Rodrigues et al., 1999). These antigens can also provide significant protection as prophylactic DNA or recombinant protein vaccines (Costa et al., 1998; Wizel et al., 1998; Fujimura et al., 2001; Ouaissi et al., 2002; Boscardin et al., 2003), and TSA-1 and Tc24 have been shown to be effective as therapeutic DNA vaccines (Dumonteil et al., 2004).

**Materials and methods**

**Experimental infection of mice**

To avoid potential genetic restriction of vaccine efficacy in inbred mice, we used outbred ICR mice, which show MHC polymorphism. Six-week-old female mice were infected by intraperitoneal injection of a lethal dose of 500 blood trypomastigotes of the H1 strain, previously isolated from a human case in Yucatán, Mexico, as before (Dumonteil et al., 2004; Zapata-Estrella et al., 2006). Infection was monitored by counting peripheral blood parasites every 3 days with a Neubauer cell, and mortality was recorded daily. For statistical comparisons, the area under the curve of parasitemia was calculated for 42 days of infection. Mice were sacrificed at the end of the acute phase, after 50 days of infection. The hearts were removed and processed for histopathologic analysis of tissue damage, and quantification of \textit{T. cruzi} parasite burden as indicators of disease progression.

**Plasmids construction and immunization**

Plasmids based on the pcDNA3.1 plasmid vector (Invitrogen, USA) and encoding \textit{T. cruzi} antigens TSA-1 (GenBank accession no. M58466) and Tc24 (U70035) have been described previously (Dumonteil et al., 2004). Plasmids p1gSPclone9 (Boscardin et al., 2003) and p154/13 (Costa et al., 1998) encode \textit{T. cruzi} antigens ASP-2-like clone 9 (SPclone9, AY186572) and trans-sialidase (TS, D50685), respectively, and are also based on the same plasmid vector. Tc52 cDNA (AF117240) was digested with BamHI and EcoRI to generate a 1900-bp fragment containing the full...
length gene and subcloned into pcDNA3.1 by standard molecular biology protocols. We also included in our study a plasmid encoding *Leishmania donovani* NH36 (Aguilar-Be et al., 2005) as an additional control for the specificity of the therapy. All plasmid DNAs were prepared using BIO-RAD Maxi-Prep kit (BIO-RAD, USA) and stored at −20 °C until use.

Infected mice were treated with two doses of plasmid DNA during the acute phase, at days 5 and 12 postinfection (Dumonteil et al., 2004). Although these times may be of limited practical usefulness, they represent an appropriate model for the rapid evaluation of vaccine candidates. Mice were anesthetized with pentobarbital and injected in the tibialis anterioris with 20 μg DNA and 45 μg of aluminum phosphate as adjuvant (Ulmer et al., 1999; Rosado-Vallado et al., 2005). Control groups of mice received 20 μg of empty pcDNA3.1 plasmid with adjuvant or saline solution.

**Histopathologic analysis**

Surviving mice were sacrificed after 50 days of infection and hearts were removed for histopathologic analysis. Samples were fixed in 10% formaldehyde and included in paraffin. Five-micrometer sections were then stained with hematoxylin and eosin, and examined for the presence of inflammation and parasites with a Nikon Eclipse E600 optical microscope at ×10 magnification. Inflammation was quantified by image analysis from four to 10 digital micrographs per animal using Multispec 3.0 (Purdue University, IN). Pixels corresponding to inflammatory cell nuclei were quantified to estimate the number of inflammatory cells per area of muscle fiber (Zapata-Estrella et al., 2006).

**Trypanosoma cruzi detection and semiquantitation**

Semiquantitation of parasite DNA was performed by competitive PCR (Roberts et al., 2000; Zapata-Estrella et al., 2006). Briefly, a parasite-specific 90-bp sequence of the β-tubulin gene was amplified from 100 ng DNA from dissected cardiac tissue using the following primers: forward 5′-ACGTTCGCTGACCTGAAAGCC-3′ and reverse: 5′-CAG GTCAGAGTTCAGCTGGC-3′. PCR amplification was performed in the presence of serial dilutions of a competing 70-bp synthetic oligonucleotide: 5′-ACGTTCGCTGACCTGAAAGCC-3′ and reverse: 5′-CAG GTCAGAGTTCAGCTGGC-3′. PCR amplification was performed in the presence of serial dilutions of a competing 70-bp synthetic oligonucleotide: 5′-ACGTTCGCTGACCTGAAAGCC-3′ and reverse: 5′-CAG GTCAGAGTTCAGCTGGC-3′. The amount of *T. cruzi* DNA was estimated by comparing band intensities of *T. cruzi* and competitor amplicons (Roberts et al., 2000; Zapata-Estrella et al., 2006).

**Statistical analysis**

Comparisons between treatment groups was performed using ANOVA, and followed, when significant, by Tukey’s post hoc test to identify the significantly different groups. The Log rank test of Kaplan–Meier survival curves was performed to assess differences in survival between groups. Correlation analysis was performed to evaluate the relationship of some variables between the different vaccine groups. All tests were performed using JMP 5.0 software (SAS Institute, Cary, NC).

**Results**

To initially evaluate the efficacy of therapeutic vaccination encoding various *T. cruzi* antigens to reduce pathology, outbred ICR mice were infected with a sublethal dose of 500 blood trypomastigotes, and treated with two doses of 20 μg of DNA vaccines encoding the selected antigens, at days 5 and 12 postinfection. The time course of the parasitemia of infected and treated mice was monitored during the acute phase of the infection (Fig. 1a and b) and the area under the curve was calculated for statistical comparisons (Fig. 1c). As expected, mice treated with saline solution or empty plasmid DNA developed very high parasitemia (Fig. 1a and c). Similarly, the parasitemia was unaffected in mice treated with plasmid DNA encoding the heterologous antigen NH36 from *Leishmania donovani* (not shown). On the other hand, mice treated with the *T. cruzi* DNA vaccines presented various profiles of parasitemia, depending on the encoded antigen. Mice treated with plgSPclone9, TS (p154/13) or a combination of both plasmids presented parasitemia not significantly different for that of control mice (Fig. 1b and c), whereas mice treated with TSA-1, Tc52 or Tc24 DNA presented significant reductions in their parasitemia compared with controls (Fig. 1a and c). ANOVA, F = 5.04, P < 0.001, Tukey: P < 0.05).

The survival of mice during the acute phase was also significantly different depending on the treatment. As shown in Fig. 2, mice treated with plasmids encoding *L. donovani* NH36, TS or ASP-2-like clone 9 presented low survival rates at 50 days postinfection, similar to that of control mice treated with saline or empty vector (Kaplan–Meier survival analysis, P > 0.05). Similarly, the survival of mice treated with a combination of both plgSPClone9 and p154/13 plasmids was not significantly different from the survival of control mice (P = 0.22). On the other hand, therapeutic vaccination with plasmid DNA encoding Tc24, TSA-1 and Tc52, respectively, resulted in significant increases in the survival of treated mice (P < 0.05). There was an overall significant inverse correlation between parasitemia (taken as the average area under the curve for each group) and survival rate at 50 days postinfection (r2 = 0.78,
$P = 0.003$), with the higher survival rate being found in the groups of mice presenting lower parasitemia.

To evaluate in more detail the extent of the control of Chagas disease progression by the therapeutic vaccines, we investigated tissue inflammation and parasite burden in the hearts of treated mice. We used digital image analysis to quantify inflammatory cell density in micrographs of cardiac tissue sections. Significant differences were observed between treatments ($\text{ANOVA } F = 12.03, P < 0.001$). At 50 days postinfection, the hearts of control mice presented an intense diffuse inflammatory reaction (Fig. 3a and b), characterized by a high density of inflammatory cells (Fig. 4). Treatment with a combination of plgSPclone 9 and p154/13 plasmids did not prevent tissue inflammation (Figs 3c and 4). Similarly, treatment with pcDNA3-Tc52 did not significantly affect the overall inflammatory cell density (Figs 3d and 4). Only treatments with TSA-1 and Tc24 DNA vaccines were able to significantly reduce cardiac tissue inflammation (Fig. 4, Tukey: $P < 0.05$), and this was mostly focal (Fig. 3e and f).

Examination of parasite burden in the heart by semi-quantitative PCR further showed that treatment with a combination of plgSPclone 9 and p154/13 plasmids had no significant effect on parasite burden, although it was slightly decreased. On the other hand, treatment with plasmid encoding the antigens TSA-1, Tc52 and Tc24 significantly reduced $T. cruzi$ parasite burden by about 2–3 log ($\text{ANOVA } F = 6.02, P = 0.001$, Tukey: $P < 0.05$). Overall, there was a significant positive correlation between parasite burden and the density of inflammatory cells ($r^2 = 0.62, P = 0.03$). An increased survival was also correlated with a lower parasite burden ($r^2 = 0.84, P = 0.009$) but not with tissue inflammation ($P = 0.13$). Taken together, these results demonstrate that DNA vaccines encoding antigens Tc24, TSA-1 and Tc52 can clearly reduce Chagas disease development at 50 days postinfection.

**Discussion**

The present comparative evaluation of therapeutic DNA vaccines against $T. cruzi$ infection demonstrates that efficacy is critically dependent on the antigen encoded. Our data first show that parasite-specific antigens are required as a vaccine encoding a $Leishmania donovani$ antigen, otherwise very effective against $Leishmania$ ssp. (Aguilar-Be et al., 2005), was completely ineffective for $T. cruzi$ therapy. This observation and the fact that the vector alone had negligible efficacy indicate that a nonspecific adjuvant-like effect of the DNA vaccine can be ruled out. Strain-specific polymorphism of the antigens may have also influenced the efficacy of the scaffold.
DNA vaccines tested here and this is a growing concern in vaccine development (Urwin et al., 2004; Kumkhaek et al., 2005), as some polymorphisms of T-cell restricted epitopes have been described, in particular for TS antigens (Martin et al., 2006), and we did not use antigen sequences derived from the same strain used for experimental infection. However, it is of key importance to identify antigens sufficiently conserved to allow for the induction of a protective/therapeutic immunity against most if not all \( T.\ cruzi \) strains, and further studies would be required to assess vaccine efficacy against different parasite strains. Alternatively, differences in the level of protein expression from the different plasmids may have affected their efficacy. This is, however, unlikely because all the vaccines were based on the same plasmid backbone and very similar levels of expression can thus be expected.

The lack of therapeutic efficacy of DNA vaccines encoding ASP-2-like and TS antigens was unexpected given the high protective efficacy of these antigens when used as prophylactic vaccines (Costa et al., 1998; Boscardin et al., 2003). Even treatment with a combination of plasmids encoding these two antigens, which could have been expected to provide an improved efficacy compared with the respective antigens alone, was unable to control disease progression. Rather, this combination of vaccines mimicked the treatment with ASP-2-like DNA alone, suggesting that this antigen may be immunodominant over TS. The contrast between the prophylactic and therapeutic efficacy of these vaccines may be due to differences in immunogenicity towards different strains of parasites as mentioned above, but may also be due to differences in plasmid doses and immunization protocols. Indeed, previous prophylactic studies used a higher plasmid dose and cardiotoxin pretreatment (Costa et al., 1998; Boscardin et al., 2003), which is known to increase vaccine efficacy.

On the other hand, Tc24, TSA-1 and Tc52 DNA vaccines provided a significant control of disease progression, as evidenced by a reduction in parasitemia, mortality and cardiac tissue inflammation. We also show for the first time that treatment with these vaccines significantly reduced parasite burden in cardiac tissue. The contrasting therapeutic efficacy of trypomastigote-specific antigens TSA-1 and TS, which both belong to the trans-sialidase family of proteins, may be related to their rather low sequence similarity (27% amino acid identity and 55% similarity), resulting in distinct T-cell epitopes (Taibi et al., 1993; Wrightsman et al., 1994; Wizel et al., 1998; Rodrigues et al., 1999). Also, some epitopes may be shared or not within this extremely large family of proteins of over 1400 members (El-Sayed et al., 2005), and thus cause potential cross-reaction with other trans-sialidases in the case of TSA-1.

Tc24 DNA seemed to induce the best therapeutic effect as it was able to induce strong reductions in all the parameters measured to evaluate disease progression. As this antigen is present in all developmental stages of \( T.\ cruzi \) (Ouaisi et al., 1992; Taibi et al., 1993), it may target both circulating and intracellular parasites, which may explain this higher efficacy in controlling disease progression. In this respect, the efficacy of Tc52 DNA vaccine may thus be related to the low but significant expression of this antigen in trypomastigotes in addition to its elevated expression in amastigotes. Taken together, these results suggest that efficacy of \( T.\ cruzi \) therapeutic vaccines may be related, in part, to their developmental stage-specificity, and constitutive antigens such as Tc24 that make it possible to target both circulating and intracellular parasites may represent better candidates. Testing of additional antigens should help clarify this point. Alternatively, the efficacy of Tc52 may be related to the fact that it is an excreted/secreted protein that is released from the parasite (Fernandez-Gomez et al., 1998), and has a potent immunomodulatory activity (Ouaisi et al., 2002). Indeed, this antigen has been proposed as a virulence factor as the targeted deletion of this gene reduced the pathogenicity of the mutant parasites (Garzon et al., 2003).

Finally, the observation of a significant correlation between parasite burden and inflammatory cell density in cardiac tissue agrees with the growing amount of evidence indicating that parasite presence and persistence results in
chronic inflammatory reactivity (Zhang & Tarleton, 1999; Monteon-Padilla et al., 2001). This strengthens the relevance of targeting the parasite for the control of Chagas disease, as decreased parasite burden may imply a better prognosis during chronic infection (Bhatia & Garg, 2005), and provide additional support for the concept of therapeutic

Fig. 3. Effect of therapeutic vaccination on cardiac tissue damage. After 50 days of infection, treated mice were sacrificed and their hearts were removed, fixed, and included in paraffin. Sections were stained with hematoxylin and eosin and observed at ×10 magnification. Representative micrographs of tissue sections from infected mice treated with saline solution (a); empty plasmid vector (b); a combination of pIGSPl9 and p154/13 DNA vaccines (c); Tc52 (d); TSA-1 (e); and Tc24 (f) DNA vaccines are shown.
vaccination aimed at reducing cardiac tissue damage. Also, the present study was limited to an acute phase model, and it would be important to confirm the present results when treatment is given during the chronic phase, when novel treatments are most needed. We previously showed that therapeutic administration of DNA encoding TSA-1 remains effective during the chronic phase (Dumonteil et al., 2004; Zapata-Estrella et al., 2006), but the therapeutic potential of other antigens at this stage remains to be optimized.

In conclusion, we show here that treatment with DNA vaccines encoding Tc52, Tc24 and TSA-1 antigens is able to reduce parasitemia, mortality, cardiac tissue inflammation and parasite burden during acute *T. cruzi* infection in mice, indicating a significant control of disease progression. These results warrant further studies of these therapeutic DNA vaccines and of the immune mechanisms that underly their efficacy, and confirm the potential of this strategy as a promising alternative or complement to conventional chemotherapy for the control of Chagas disease.

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