Immunotherapy of *Trypanosoma cruzi* Infection with DNA Vaccines in Mice

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Chagas’ disease, or American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi* and has a widespread distribution in the Americas. An estimated 16 million to 18 million persons are infected, and close to 100 million people are at risk of infection. Transmission to humans occurs primarily through blood-sucking reduvid bugs, which deposit infective feces on the skin when they are feeding, but it may also occur through blood transfusion (30, 51).

The disease is characterized by an initial acute phase during which flagellated parasites (trypanastigotes) multiply in the blood, followed by an indeterminate phase with very low parasitemia and no apparent pathology. Susceptible hosts then enter a chronic phase with increasing tissue damage, mostly in cardiac and skeletal muscle tissues but sometimes also in the liver, spleen, colon, or esophagus, that progressively leads to cardiac and skeletal muscle tissue damage, mostly in cardiac and skeletal muscle tissues but sometimes also in the liver, spleen, colon, or esophagus, that progressively leads to cardiac failure and death (2, 5, 36). There is still considerable debate about the mechanisms involved in the pathology (14, 19, 44); some evidence indicates that damage is associated with the presence and replication of intracellular amastigotes in host tissues, while other studies have shown that autoimmunity induced by parasite antigens mimicking host proteins is responsible for tissue damage. Indeed, standard histopathological analysis of tissues from chronically infected hosts shows that there are abundant inflammatory cells but few or no amastigotes nests, suggesting that the pathology may develop in an almost complete absence of parasites. These observations have been associated with identification of several cross-reacting antibodies and T cells to support the autoimmune hypothesis (15, 24). However, more sensitive parasite detection techniques have confirmed that parasites persist in tissues and correlated this persistence with tissue damage (31, 32). Furthermore, it is well accepted that a T helper 1 (Th1) type of immune response is required for clearing the parasite from infected mice (18, 28, 33, 38, 42, 49), while such a response has also been associated with the intensity of the pathology during the chronic phase in humans (1, 16). All this controversy has interfered substantially with the development of immunotherapy and preventive vaccines as there is no clear agreement concerning whether the immune response should be stimulated (to eliminate the parasite) or inhibited (to avoid autoimmunity).

Nonetheless, several studies have focused on characterization of parasite antigens which may be used as vaccine candidates. Some recombinant or purified antigens, such as paraglalel rod proteins, trans-sialidase, trypomastigote excretory-secretory antigens (including Tc24), glycoprotein 82, and trypomastigote surface antigen 1 (TSA-1), have shown promise because they induce significant protection in some mouse models (6, 28, 38, 52). More recently, DNA vaccines encoding some of these antigens have also been tested. Because DNA vaccines have strong potential for the immunotherapy of *T. cruzi* infection and may provide new alternatives for the control of Chagas’ disease.
KMP11-HSP70 fusion protein (35) leads to a Th1-type immune response that is protective in the case of TSA-1, ASP-1, ASP-2, or KMP11-HSP70. Similarly, immunization with a plasmid encoding the complement regulatory protein, but not the recombinant protein, can protect against challenge (40). These data support the idea that using DNA vaccines may be a better strategy than using recombinant vaccines against T. cruzi.

A very attractive feature of DNA vaccines is that they may also be used for immunotherapy once an infection has been initiated. This therapeutic potential has been observed against various pathogens, including human immunodeficiency virus (7), rabies virus (26), Mycoplasma pulmonis (23), Mycobacterium tuberculosis (27), Leishmania major (17), and Schistosoma mansoni (11). These studies demonstrated that administration of a DNA vaccine (alone or in combination with additional drug therapy in the case of rabies virus and Schistosoma) could clear or greatly reduce an ongoing infection by one of these pathogens.

In the case of T. cruzi, drug therapy relies on nitrofurans (such as nifurtimox, now discontinued) or nitroimidazoles (such as benznidazole). However, these drugs have little efficacy (limited to early stages of the infection), and they have serious side effects, so that efforts are needed to identify new treatments (20, 41, 47). Indeed, Chagas' disease is one of the most neglected diseases in terms of development of new drugs (45). Thus, we investigated whether DNA vaccine immunotherapy could be effective in controlling an ongoing T. cruzi infection in mice. We tested DNA plasmids encoding T. cruzi antigens TSA-1 and Tc24, which had previously been shown to be highly immunogenic and to provide good protection as prophylactic DNA and recombinant protein vaccines, respectively (43, 50). We report here the first data showing that DNA vaccine immunotherapy may be a promising strategy for control of T. cruzi infection.

MATERIALS AND METHODS

Experimental infection of mice. T. cruzi strains H1 (low virulence) and H4 (high virulence), previously isolated from human cases in Yucatán, Mexico (4), and maintained in the laboratory by monthly passages in CD1 mice, were used for infection. Two distinct experimental models of infection were used in this study. First, we used BALB/c mice as a model of acute lethal infection. Four- to six-week-old BALB/c mice were infected by intraperitoneal injection of a lethal dose, $5 \times 10^7$ blood trypomastigotes (strain H4). The development of infection was monitored as described above for up to 140 days, when surviving mice were sacrificed. Spleen, liver, heart, and skeletal muscle tissues were removed and processed for histopathological analysis and T. cruzi parasite detection (see below).

Second, as a model of chronically infected mice, we used 4- to 6-week-old CD1 mice that were infected intraperitoneally with a low dose, $5 \times 10^6$ blood trypomastigotes (strain H1). The infection was monitored as described above for up to 140 days, when surviving mice were sacrificed. Spleen, liver, heart, and skeletal muscle tissues were removed and processed for histopathological analysis and T. cruzi parasite detection.

The experimental protocol was approved by the Bioethics Committee of the Universidad Autónoma de Yucatán, and all animal experiments were carried out according to the established guidelines. All mice were bred and cared for at the animal facilities of our research center.

Plasmid construction. The cDNA encoding T. cruzi TSA-1 (kindly provided by J. Manning, Molecular Biology and Biochemistry, University of California, Irvine) was digested with BamHI and EcoRI to generate a 1,900-bp fragment corresponding to the 5' region (nucleotides 1 to 1851) (50) and was subcloned into pcDNA3 (Invitrogen, Carlsbad, Calif.) by using standard molecular biology techniques.

FIG. 1. Parasitemia and survival of BALB/c mice infected with a lethal dose of T. cruzi and treated with DNA vaccines. Mice were infected with $5 \times 10^7$ T. cruzi parasites (strain H4) and treated on days 5 and 12 (arrows) with intramuscular injections of 100 µg of plasmid DNA encoding TSA-1 (○) or Tc24 (■), control pcDNA3 plasmid (□), or a saline solution (◇). (A and B) Infection monitored by measuring parasitemia. The values are means ± standard errors of the means for 3 to 15 mice per group. (C) Survival of infected and treated BALB/c mice.
FIG. 2. Histopathological analysis of cardiac tissue from BALB/c mice infected with a lethal dose of *T. cruzi* and treated with DNA vaccines. Cardiac tissue was examined on days 40 to 50 postinfection (A, B, C, E, and G) or on day 140 postinfection (D, F, and H). Infected mice treated with a saline solution (A) or the control pcDNA3 plasmid (B) died by days 40 to 45 and had extensive inflammatory infiltrates, scattered to abundant amastigote nests (arrows), and some fibrosis and necrosis. Mice treated with 100 μg of DNA vaccine encoding TSA-1 on days 5 and 12 (C) had only mild focal inflammation (asterisk). When treatment was delayed and administered on days 10 and 17 postinfection (E) or on days 15 and 22 postinfection (G), the inflammation level appeared to be intermediate between that of treated mice and that of untreated mice. On day 140 after infection, BALB/c mice treated with TSA-1 DNA on days 5 and 12 (D) or on days 10 and 17 (F), as well as mice treated with Tc24 DNA (H), all had very mild and diffuse inflammatory infiltrates, and amastigote nests were undetectable except in one mouse.
We initially administered DNA vaccines encoding TSA-1 and Tc24 antigens during the acute phase of infection in highly susceptible BALB/c mice and evaluated the efficacy of DNA vaccine immunotherapy by monitoring the parasitemia and mortality of infected mice. As expected, mice that were inoculated with a lethal dose, $5 \times 10^4$ trypomastigotes, and received only the saline solution rapidly developed a high level of parasitemia (Fig. 1A), and all died by day 45 postinfection (Fig. 1C). Similarly, mice that were treated with the empty plasmid pCNA3 also developed a high level of parasitemia, albeit slightly delayed, and all died by day 50 (Fig. 1A and C). In contrast, mice that were treated with two injections of plasmid DNA encoding TSA-1, on days 5 and 12 postinfection, had a lower level of parasitemia, and circulating parasites became undetectable by direct microscopic examination by day 42 (Fig. 1A). In addition, more than 70% of the pcDNA3-TSA1-treated mice survived the acute phase of the infection (Fig. 1C). Immunotherapy with the plasmid encoding Tc24 also controlled the infection, as indicated by the lower level of parasitemia and the survival of $100\%$ of the mice treated with this plasmid (Fig. 1B and C). Histopathological analysis of cardiac tissue at the end of the acute phase (days 40 to 50 postinfection) indicated that control mice that received the saline solution or the empty plasmid had extensive inflammation with diffuse infiltration of mononuclear cells (Fig. 2A and B). Some necrosis and fibrosis were observed, and scattered to abundant T. cruzi amastigote nests were detected. On the other hand, mice treated with plasmid DNA encoding TSA-1 had only mild focal inflammation and tissue damage (Fig. 2C), and only rare amastigote nests were detected.

We then tested whether the timing of plasmid DNA administration was crucial for efficacy of the treatment. We thus delayed initiation of the immunotherapy until 10 and 15 days postinfection (Fig. 3). This delay in injection of plasmid DNA by using the TC1 and protocols, yielding pCNA3-TSA1. Tc24 DNA was amplified by PCR from T. cruzi DNA by using forward primer 5'-GTATGGTGACCCAGTGCCCAACGAG-3' and reverse primer 5'-CGCGAATTCACAAGCAGTCCTGCGG-3'. PCR products were digested with BamHI and Kpnl and also cloned into pCNA3, yielding pCNA3-Tc24. Plasmids were checked by extensive restriction digestion and sequencing prior to injection. Plasmid DNA was prepared by using a Bio-Rad Maxi-Prep kit (Bio-Rad, Hercules, Calif.) and was stored at -20°C until it was used.

**Immunotherapy of infected mice.** Immunotherapy with DNA vaccines was tested in both the acute and chronic infection models described above. For treatment of an acute lethal infection, BALB/c mice were anesthetized with pentoobarbital on day 5 postinfection and treated by intramuscular injection in both the acute and chronic infection models described above. For treatment of an acute lethal infection, BALB/c mice were anesthetized with pentoobarbital on day 5 postinfection and treated by intramuscular injection in both the acute and chronic infection models described above.

**RESULTS**

**Efficacy of DNA vaccine immunotherapy administered during the acute phase.** We initially administered DNA vaccines encoding TSA-1 and Tc24 antigens during the acute phase of infection in highly susceptible BALB/c mice and evaluated the efficacy this immunotherapy by monitoring the parasitemia and mortality of infected mice. As expected, mice that were inoculated with a lethal dose, $5 \times 10^4$ trypomastigotes, and received only the saline solution rapidly developed a high level of parasitemia (Fig. 1A), and all died by day 45 postinfection (Fig. 1C). Similarly, mice that were treated with the empty plasmid pCNA3 also developed a high level of parasitemia, albeit slightly delayed, and all died by day 50 (Fig. 1A and C). In contrast, mice that were treated with two injections of plasmid DNA encoding TSA-1, on days 5 and 12 postinfection, had a lower level of parasitemia, and circulating parasites became undetectable by direct microscopic examination by day 42 (Fig. 1A). In addition, more than 70% of the pCNA3-TSA1-treated mice survived the acute phase of the infection (Fig. 1C). Immunotherapy with the plasmid encoding Tc24 also controlled the infection, as indicated by the lower level of parasitemia and the survival of $100\%$ of the mice treated with this plasmid (Fig. 1B and C). Histopathological analysis of cardiac tissue at the end of the acute phase (days 40 to 50 postinfection) indicated that control mice that received the saline solution or the empty plasmid had extensive inflammation with diffuse infiltration of mononuclear cells (Fig. 2A and B). Some necrosis and fibrosis were observed, and scattered to abundant T. cruzi amastigote nests were detected. On the other hand, mice treated with plasmid DNA encoding TSA-1 had only mild focal inflammation and tissue damage (Fig. 2C), and only rare amastigote nests were detected.
pcDNA3-TSA1 resulted in progressively higher levels of parasitemia (Fig. 3A). Nonetheless, the parasitemia remained controlled and eventually became undetectable, as it did when treatment was initiated on day 5 postinfection. Accordingly, 70 to 100% of the treated mice survived the acute phase of infection (Fig. 3B). Histopathological analysis also showed that there was a reduction in tissue damage on day 50 postinfection when even treatment was delayed, although the infiltrates of inflammatory cells appeared to be more abundant and diffuse than those observed in mice treated on day 5 postinfection (Fig. 2E and G). Taken together, these data indicate that immunotherapy with a DNA vaccine encoding TSA-1 or Tc24 can induce short-term (up to 50 days) control of a lethal T. cruzi infection in BALB/c mice.

Because of the complex nature of T. cruzi pathology, such short-term control of the acute phase may not be sufficient to prevent development of the more damaging chronic phase of the disease. We thus investigated the long-term effects of DNA vaccine immunotherapy by evaluating cardiac tissue damage and T. cruzi persistence in treated mice at 140 days postinfection. As circulating blood parasites are difficult to detect by microscopic examination at this stage, we used PCR to test for the presence of the parasite. We did not detect T. cruzi kDNA in the blood of treated mice at this stage. Histopathological analysis of cardiac tissue revealed mild and diffuse inflammatory infiltrates even when treatment had been delayed, and rare amastigote nests were present in only one mouse (Fig. 2D, F, and H). However, PCR analyses of the cardiac biopsies revealed that all mice treated with plasmid DNA encoding TSA-1 (on days 5 and 12 or on days 10 and 17) were positive for T. cruzi kDNA, whereas only one-third of the mice treated with Tc24 showed evidence of T. cruzi kDNA ($\chi^2 = 6.19; P = 0.04$) (Table 1). Finally, we evaluated the presence of live infective parasites in the hearts of the treated mice by inoculating heart tissue homogenates from these animals into naïve BALB/c mice and monitoring the parasitemia. When mice were treated with the TSA-1 plasmid on days 5 and 12 postinfection, only one-third of the animals still had live parasites in the cardiac tissues on day 140. When treatment was initiated 10 days postinfection, three-quarters of the mice had live parasites (Table 1). All of the mice treated with plasmid DNA encoding Tc24 appeared to be free of live T. cruzi parasites, as naïve mice injected with heart homogenates from these mice remained uninfected (Table 1). However, the differences were not statistically significant ($\chi^2 = 5.14; P = 0.07$). Taken together, these data indicate that DNA vaccine immunotherapy during the acute phase had long-term effects that reduced the development of chronic chagasic cardiomyopathy in treated mice.

**Efficacy of DNA vaccine immunotherapy administered during the chronic phase.** We then tested the efficacy of DNA vaccine immunotherapy during the chronic phase of infection. We used CD1 mice that were infected with a low dose of parasites, which allowed more than 75% of these mice to survive (Table 1). When mice were treated with the TSA-1 plasmid on days 5 and 12 postinfection, only one-third of the animals still had live parasites in the cardiac tissues on day 140. When treatment was initiated 10 days postinfection, three-quarters of the mice had live parasites (Table 1). All of the mice treated with plasmid DNA encoding Tc24 appeared to be free of live T. cruzi parasites, as naïve mice injected with heart homogenates from these mice remained uninfected (Table 1). However, the differences were not statistically significant ($\chi^2 = 5.14; P = 0.07$). Taken together, these data indicate that DNA vaccine immunotherapy during the acute phase had long-term effects that reduced the development of chronic chagasic cardiomyopathy in treated mice.

**TABLE 1. Parasitological study on day 140 after infection of BALB/c mice treated during the acute phase of the infection**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of treatment (days postinfection)</th>
<th>Parasite DNA in blood</th>
<th>Inflammation</th>
<th>Amastigote nests</th>
<th>Parasite DNA in heart tissue</th>
<th>Live parasites isolated from heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3-TSA1</td>
<td>5</td>
<td>0/3</td>
<td>+</td>
<td>− to +</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0/4</td>
<td>++</td>
<td>− to +</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td>pcDNA3-Tc24</td>
<td>5</td>
<td>0/3</td>
<td>+</td>
<td>−</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>5</td>
<td>ND</td>
<td>+++</td>
<td>+ to +++</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>ND</td>
<td>+++</td>
<td>+ to +++</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

- Number of mice with blood positive for T. cruzi as determined by PCR/number of mice analyzed.
- Histological detection of amastigote nests in heart tissue sections. −, undetectable; +, rare; ++, moderate; ++++, abundant.
- Number of mice with T. cruzi kDNA in heart tissue as determined by PCR/number of mice analyzed ($\chi^2 = 6.19; P = 0.04$).
- Number of mice from which live T. cruzi parasites were isolated from heart tissue/number of mice analyzed ($\chi^2 = 5.14; P = 0.07$).
- Tissues were obtained from the mice on day 5 postinfection.
- ND, not done.

**TABLE 2. Parasitological study on day 140 postinfection of CD1 mice treated during the chronic phase of the infection**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of treatment (days postinfection)</th>
<th>Survival</th>
<th>Parasite DNA in blood</th>
<th>Inflammation</th>
<th>Amastigote nests</th>
<th>Parasite DNA in heart tissue</th>
<th>Live parasites isolated from heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3-TSA1</td>
<td>70</td>
<td>7/7</td>
<td>0/7</td>
<td>+</td>
<td>−</td>
<td>7/7</td>
<td>7/7</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>70</td>
<td>4/6</td>
<td>0/4</td>
<td>++</td>
<td>−</td>
<td>4/4</td>
<td>ND</td>
</tr>
<tr>
<td>Saline</td>
<td>70</td>
<td>3/7</td>
<td>0/3</td>
<td>+++</td>
<td>−</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

- Number of mice surviving on day 140/number of mice studied ($\chi^2 = 7.23; P = 0.027$).
- Number of mice with blood positive for T. cruzi as determined by PCR/number of mice analyzed.
- Histological detection of amastigote nests in heart tissue sections. −, undetectable.
- Number of mice with detectable T. cruzi DNA in heart tissue/number of mice analyzed.
- Number of mice from which live T. cruzi parasites were isolated from heart tissue/number of mice analyzed.
- ND, not done.
survive the acute phase of the infection (data not shown). Surviving mice then received plasmid DNA encoding TSA-1 or a saline treatment on day 70 postinfection and were monitored up to day 140 postinfection. Both groups of mice had no circulating parasites in their blood at this stage, as assessed by PCR (Table 2). All seven mice that received the pcDNA3-TSA1 vaccine treatment survived up to day 140, whereas only four (66%) of the six pcDNA3-treated control mice and three (42%) of the seven saline solution-treated control mice survived ($\chi^2 = 7.23; P = 0.027$). In addition, histopathological analysis indicated that control mice that received the saline solution or pcDNA3 had extensive diffuse inflammation and chronic myocarditis (Table 2 and Fig. 4A). On the other hand, mice treated with the DNA vaccine encoding TSA-1 had mild focal inflammation (Table 2 and Fig. 4B). As expected, we did not observe amastigote nests in cardiac tissue sections from either group, confirming the low sensitivity of the standard technique for histopathologic detection of parasites. However, we detected *T. cruzi* kDNA and live parasites in cardiac biopsies of all treated and control mice (Table 2). Taken together, these data suggest that DNA vaccine immunotherapy during the chronic phase may reduce but not eliminate *T. cruzi* parasites and thus limit progression of chronic chagasic cardiomyopathy.

**DISCUSSION**

DNA vaccines have been described as a powerful alternative for development of preventive vaccines against a wide variety of infectious and chronic diseases (25). Interestingly, they also have been shown to have potential for immunotherapy against several pathogens (7, 11, 17, 23, 26, 27). Given the lack of effective therapeutic drugs for use against *T. cruzi*, we investigated the efficacy of DNA vaccine immunotherapy against *T. cruzi* infection.

We used DNA vaccines encoding *T. cruzi* antigens TSA-1 and Tc24, because both of these antigens were reported to be highly immunogenic and protective in previous vaccine studies (43, 50). In addition, TSA-1 has been shown to induce significant protection as a preventive DNA vaccine (13, 50). To our knowledge, this is the first report which clearly demonstrates that intramuscular injection of only two doses of plasmid DNA encoding TSA-1 or Tc24 during the acute phase of infection is sufficient to induce control of an otherwise lethal *T. cruzi* infection. Indeed, such treatments dramatically reduced the parasitemia and cardiac tissue damage and resulted in increased survival of the infected animals. Most noticeable was the ability of DNA vaccine immunotherapy to reduce the levels of parasites to levels below PCR- or infection-detectable levels in some of the treated mice in which we were unable to detect parasite DNA or live parasites in cardiac biopsies. The efficacy of the treatment remained significant when its administration was delayed. However, the mice treated later during the acute phase had slightly higher levels of parasitemia and more extensive inflammatory damage than the mice treated soon after infection. Thus, when the immunotherapy was administered to chronically infected mice (70 days postinfection), we found that it was too late to eliminate the parasites. Nonetheless, treatment during the chronic phase remained sufficiently efficient to reduce tissue damage and chronic disease progression, and it significantly improved survival. Thus, the DNA vaccine immunotherapy was effective and beneficial when it was administered at any stage of infection.

The mechanisms of action of our DNA vaccine immunotherapy are not known, but our results are consistent with possible
reorientation and/or potentiation of the immune response from a nonprotective Th2 response to a protective Th1 response (21). Indeed, it is accepted that DNA vaccines have a significant Th1 bias (10, 22, 39), and a DNA vaccine encoding TSA-1 has been shown to induce strong CD8+ responses (50). Also, reorientation of the immune response has been observed in the case of DNA vaccine therapy against Leishmania or Mycobacterium infections (17, 27). A shift in the immune response may have to occur early enough in the infection to eliminate T. cruzi and avoid dissemination or establishment in host target tissues, which may explain the lower efficacy of the treatment when it is delayed. Understanding these immune mechanisms and their kinetics should provide important clues for improving and optimizing the efficacy of this therapy, and studies to do this are under way.

An additional important observation is that our results support the idea that parasite persistence (3) rather than autoimmunity (12) may cause chronic inflammation and tissue damage and thus disease. Thus, stimulation of the immune response, and probably stimulation of a Th1-type response, did not result to exacerbation of the disease. This contradicts the results of previous studies of chagasic patients which suggested that chronic chagasic cardiomyopathy is associated with a Th1 response (1, 16), but it is in agreement with other suggestions that control of T. cruzi infection should be aimed at parasite elimination through drug therapy or stimulation of the immune response (46). Quantification of parasites following immunotherapy should clarify this point and establish a possible correlation between parasite load and inflammatory damage.

In conclusion, we showed here that DNA vaccine immunotherapy can effectively control T. cruzi infection and significantly reduce the progression of chronic chagasic cardiomyopathy in mice. The efficacy of this strategy appears to be superior to or possibly comparable to the efficacy of chemotherapy, and the strategy has the major advantage of requiring only two doses, whereas drug treatments rely on daily doses for a prolonged time (29, 47). The treatment described here may thus represent a promising alternative for Chagas’ disease therapy, alone or in combination with other drug treatments.

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