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Protective Immunity Against *Trypanosoma cruzi* Infection in a Highly Susceptible Mouse Strain After Vaccination with Genes Encoding the Amastigote Surface Protein-2 and *Trans*-Sialidase

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ABSTRACT

Protective immunity against lethal infection is developed when BALB/c or C57BL/6 mice are immunized with plasmids containing genes from the protozoan parasite *Trypanosoma cruzi*. However, genetic vaccination of the highly susceptible mouse strain A/Sn promoted limited survival after challenge. This observation questioned whether this type of vaccination would be appropriate for highly susceptible individuals. Here, we compared the protective efficacy and the immune response after individual or combined genetic vaccination of A/Sn mice with genes encoding *trans*-sialidase (TS) or the amastigote surface protein-2 (ASP-2). After challenge, a significant proportion of A/Sn mice immunized with either the *asp-2* gene or simultaneously with *asp-2* and *ts* genes, survived infection. In contrast, the vast majority of mice immunized with the *ts* gene or the vector alone died. Parasitological and histological studies performed in the surviving mice revealed that these mice harbored parasites; however, minimal inflammatory responses were seen in heart and striated muscle. We used this model to search for an *in vitro* correlation for protection. We found that protective immunity correlated with a higher secretion of interferon-γ by spleen cells on *in vitro* restimulation with ASP-2 and the presence of ASP-2–specific CD8 cells. Depletion of either CD4 or CD8 or both T-cell subpopulations prior to the challenge rendered the mice susceptible to infection demonstrating the critical contribution of both cell types in protective immunity. Our results reinforce the prophylactic potential of genetic vaccination with *asp-2* and *ts* genes by describing protective immunity against lethal *T. cruzi* infection and chronic tissue pathology in a highly susceptible mouse strain.

OVERVIEW SUMMARY

Here, we report that after challenge with the protozoan parasite *Trypanosoma cruzi*, a significant proportion of A/Sn mice immunized with either the gene encoding the amastigote surface protein-2 (ASP-2) or simultaneously with genes encoding ASP-2 and *trans*-sialidase (TS) survived infection. In contrast, the vast majority of mice immunized with the *ts* gene or the vector alone died. Histologic analysis of surviving mice revealed a reduced inflammatory response in heart and striated muscle. The higher protective efficacy observed was associated with higher *in vitro* interferon-γ secretion by ASP-2–specific CD4 and CD8 T cells. The participation of CD4 and CD8 T cells in protective immunity of DNA-vaccinated mice was confirmed by depletion of these subpopulations prior to challenge with infective parasites. Our results reinforce the prophylactic potential of genetic vaccination with *asp-2* and *ts* genes by describing

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protective immunity against lethal *T. cruzi* infection and chronic tissue pathology in a highly susceptible mouse strain.

INTRODUCTION

THE OBLIGATORY INTRACELLULAR PROTOZOAN PARASITE *Trypanosoma cruzi* is the etiologic agent of Chagas' disease, a major health problem in Latin America. Currently, there are more than 15 million people chronically infected with an estimate of 40 million at risk of infection. The annual incidence of new cases per year is more than 700,000 and the death toll is approximately 45,000 (reviewed by Morel and Lazdins, 2003). Currently available chemotherapy is far from efficient and its efficacy varies widely according to the infection status (acute or chronic) and the endemic region. The prospect for development of new drugs is slight, with only a single new compound being tested in phase 1/2 trials (reviewed by Urbina and Docampo, 2003). The poor prospect for prevention and treatment raises the possibility that immune interventions, such as immunization, could be useful as an additional strategy to reduce disease spreading and improve treatment efficacy.

Plasmids containing different *T. cruzi* genes have been used successfully to elicit protective immunity against experimental infection (Costa *et al*., 1998; Wizel *et al*., 1998; Sepulveda *et al*., 2000; Fujimura *et al*., 2001; Planelles *et al*., 2001; Garg and Tarleton, 2002; Katae *et al*., 2002; Boscardin *et al*., 2003; Fralish and Tarleton, 2003; Miyahira *et al*., 2003). Although most studies on genetic vaccination against experimental Chagas' disease performed so far have focused on the prophylactic properties of plasmids expressing *T. cruzi* genes, a recently published study has extended the effectiveness of genetic vaccination by providing compelling evidence that this strategy may be useful for gene therapy against established chronic heart pathology (Dumonteil *et al*., 2004).

In our initial studies on genetic vaccination against experimental Chagas' disease, we used the gene encoding *T. cruzi trans*-sialidase (TS). Protective immunity could be reproducibly observed in vaccinated BALB/c mice (Costa *et al*., 1998; Fujimura *et al*., 2001). In spite of the successful vaccination in this mouse strain, we failed to replicate this protective immunity in a mouse strain (A/Sn) highly susceptible to *T. cruzi* infection (Vasconcelos *et al*., 2003). The lack of protective immunity observed after genetic immunization of this highly susceptible mouse strain raised questions as to whether this type of vaccination strategy would be appropriate for more susceptible individuals. We consider this subject extremely relevant because individual genetic susceptibility may be a key issue in the immunopathology of Chagas' disease (Marinho *et al*., 2004).

Recently, different groups have described protective immunity against *T. cruzi* infection in BALB/c or C57BL/6 mice, induced by genetic vaccination with plasmids expressing the amastigote surface protein-2 (ASP-2; Garg and Tarleton, 2002; Boscardin *et al*., 2003; Fralish and Tarleton, 2003). The description of immunoprotective properties of this gene/antigen allowed us to reevaluate whether genetic vaccination could be performed in a highly susceptible mouse strain. Here we report an improvement on the protective efficacy of DNA vaccination by immunizing highly susceptible A/Sn mice individually with a plasmid harboring the gene *asp-2* or simultaneously with plasmids containing *asp-2* and *ts* genes from *T. cruzi*.

MATERIALS AND METHODS

Parasites and mice

Bloodstream trypomastigotes of the Y strain were obtained from 7-day-infected A/Sn mice. The female 5- to 8-week old A/Sn mice used in this study were purchased from the University of São Paulo, SP, Brazil. Mice were challenged intraperitoneally with 200 bloodstream trypomastigotes of the Y strain. Parasite development was monitored in the blood using a standard method (Krettli and Brener, 1976).

Plasmid generation, purification and mouse immunization

The plasmids, denominated p154/13 (containing the *ts* gene) and pIgSPclone9 containing the *asp-2* gene, were created, purified, and injected as previously described by Costa *et al*. (1998) and Boscardin *et al*. (2003). Briefly, both tibialis anterioris muscles were injected with 3.5 μg of cardiotoxin (Sigma, St. Louis, MO). Five days later, 5 to 100 μg of plasmid DNA was injected intramuscularly at the same sites as the previous cardiotoxin injections (a total of 10–200 μg of plasmid per mouse). Each mouse received four intramuscular doses of 10 to 200 μg of plasmid DNA injected at 0, 3, 5, and 7 weeks. Two weeks after immunization with the plasmids, blood was collected from the tail and the sera were analyzed for the presence of antibodies to TS or ASP-2 recombinant proteins. Three weeks after the last dose, mice were challenged as described above.

Histopathologic analysis and hemoculture

Tissue specimens were collected from DNA vaccinated and infected mice 100 days after challenge. Tissue sections were stained with hematoxylin-eosin and analyzed by optical microscopy. Six nonconsecutive slides from the heart and the quadriceps of each mouse were analyzed (Marinho *et al*., 1999).

Aliquots of 0.1 ml of blood were collected and cultured in triplicate, at 28°C for 1 month, in axenic liver infusion tryptose medium. Parasite growth was monitored microscopically every week.

Detection of antibodies against recombinant TS or ASP-2

Recombinant TS or ASP-2 (His-65 kDa) proteins were produced in *Escherichia coli* as previously described (Boscardin *et al*., 2003; Vasconcelos *et al*., 2003). Sera from immunized and control mice were diluted 1000 times.

Cell-mediated immune response assays

Two weeks after the last immunization, cells were obtained from the spleens of the immunized animals. After red blood cells lyses and three washes, spleen cells were ressuspended in 1 ml of cell culture medium. Cell concentration was adjusted to 5×10^6 cells per milliliter and cultivated in flat-bottom 96-

well plates (Corning, Corning, NY) in a final volume of 200 μl. Recombinant protein was added to the cultures at final concentrations ranging from 0.001 to 10 μg/ml. Purified monoclonal antibody (mAb) anti-CD4 (Gk1.5, rat IgG2b), anti-CD8 (clone 2.43, rat IgG2b), or rat IgG (Sigma) antibodies were added at a final concentration of 10 μg/ml. After 3 days, the supernatants were collected and the cytokine concentration was estimated by capture enzyme-linked immunosorbent assay (ELISA) using antibodies purchased from Pharmingen (San Diego, CA) exactly as described (Rodrigues *et al*., 1999).

The enzyme-linked immunospot (ELISPOT) assay was performed essentially as described earlier (Boscardin *et al*., 2003). Responder cells were obtained from spleens of mice immunized with plasmids p154/13 and pIgSPclone 9 or pcDNA3. These cells were used at a concentration of 5×10^6 cells per milliliter. Depletion of T-cell subpopulations was obtained by treating 10^7 cells with 100 μg of anti-CD4 or anti-CD8 followed by rabbit complement (Rodrigues *et al*., 1999).

Antigen presenting cells were prepared by irradiating fibroblast L 929 cells for 45 min. To these cells, recombinant replication-defective adenovirus expressing TS or ASP-2 proteins was added or not. The detailed genetic, biochemical, and immunologic characterization of these recombinant viruses will be published elsewhere (A.M.V. Machado, C. Claser, R.T. Gazzinelli, M.M. Rodrigues, and O. Bruña-Romero, manuscript in preparation). The final viral concentration was 5×10^7 per milliliter representing approximately 50 virus particles per L cell. After 2 hr, virus transfected and nontransfected L cells were washed, counted, and their concentration adjusted to 10^6 cells per milliliter in complete culture medium. One hundred microliters of suspension-containing responders or antigen presenting cells were pipetted to each well. The plates were incubated in static conditions for 24 hr at 37°C in an atmosphere containing 5% CO_2.

In vivo *depletion of CD4 and CD8 T cells*

The hybridomas secreting mAb anti-CD4 (GK1.5) or anti-CD8 (53.6.7, rat IgG2a) were purchased from American Type

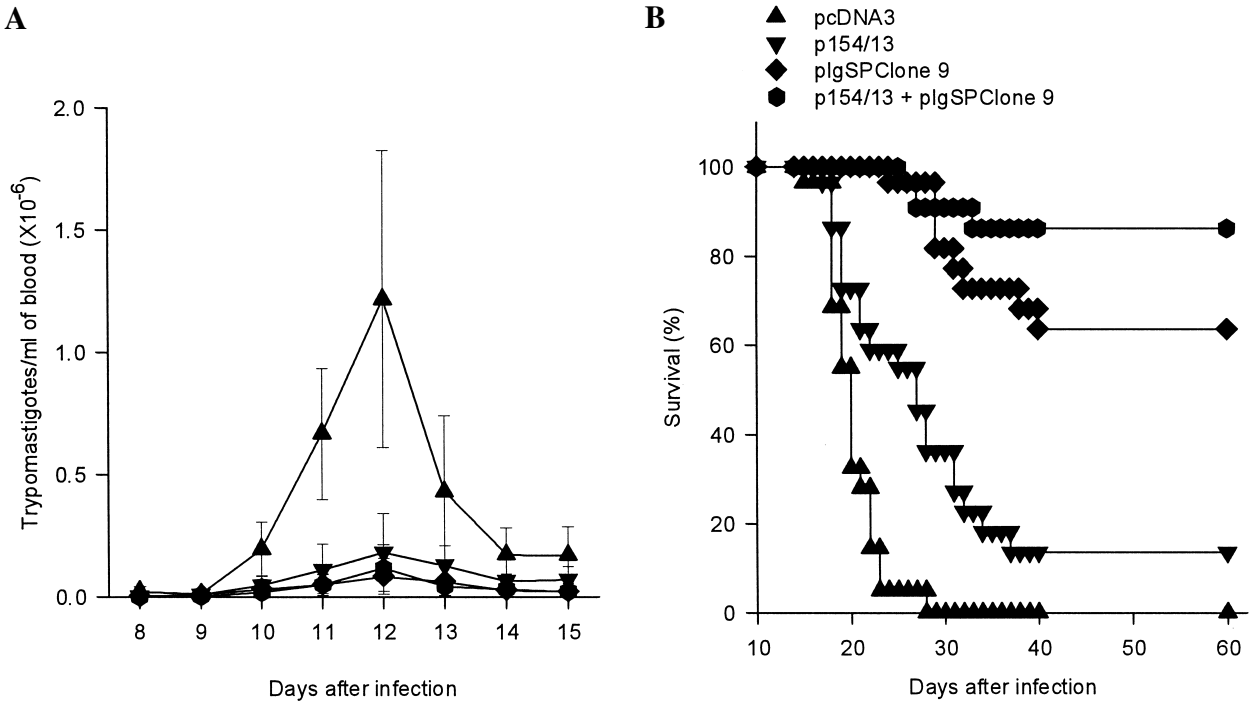

FIG. 1. Trypomastigote-induced parasitemia in mice immunized with plasmids p154/13 and pIgSPclone9 administered individually or simultaneously. Groups of mice were immunized with four doses of plasmids pcDNA3, p154/13 or pIgSPclone9. Each dose consisted of 200 μg of plasmid DNA injected at 0, 3, 5, and 7 weeks. A fourth group of mice was injected with four doses of a mixture containing, in each dose, 100 μg of p154/13 and 100 μg of pIgSPclone9. Three weeks after the last dose, mice were challenged intraperitoneally with 200 bloodstream trypomastigotes of the Y strain. **A**: The course of infection was estimated by the number of trypomastigotes per milliliter of blood. The results represent the mean of 6 mice $\pm$ standard deviation (SD). The value of the peak parasitemia observed for each mouse was used for statistical comparison using one-way analysis of variance (ANOVA) the Tukey HSD test. There was a significant reduction in peak parasitemia in animals immunized either with plasmids p154/13 or pIgSPclone9 or both compared to animals immunized with the control plasmid pcDNA3 ($p < 0.05$ in all cases). The value of peak parasitemia of mice immunized with either p154/13 or pIgSPclone9, or both plasmids were not statistically different ($p > 0.05$). Results are representative of three independent experiments. **B**: The graph shows Kaplan-Meier curves for survival of 22 mice per group. This number represents pooled results from 3 experiments with 6 or 8 mice per group. Animals immunized with p154/13 (▼) survived longer than control mice injected with pcDNA3 (▲, $p < 0.0001$, log rank test). A significant delay in mouse mortality was observed in mice injected with pIgSPclone9 alone (◆) or mixed with p154/13 (●) compared to groups of mice that received either pcDNA3 or p154/13 ($p < 0.0001$). There was no statistically significant difference between the survival curves of mice vaccinated only with pIgSPclone9 alone or mixed with p154/13 ($p > 0.05$, $p = 0.10$).

Culture Collection (Manassas, VA). At days 3 and 4 before challenge with trypomastigotes, mice immunized with the mixture of p154/13 and pIgSPclone9 were treated intraperitoneally with a dose of 1 mg/d of rat IgG, anti-CD4, anti-CD8 or anti-CD4, and anti-CD8. Seven days after challenge, each mouse received one more dose of 1 mg of rat IgG, anti-CD4, anti-CD8 or anti-CD4, and anti-CD8. The efficacy of depletion was estimated by flow cytometry analysis of spleen cells. Depletion of CD4 or CD8 spleen cells was commonly higher than 98% or 96%, respectively.

Statistical analysis

The values of peak parasitemias of each individual mouse were log transformed before being compared by one-way analysis of variance (ANOVA) and Tukey HSD tests. The log rank test was used to compare the mouse survival rate after challenge with *T. cruzi*. The OD_{492} values obtained by ELISA using sera from mice immunized with the different plasmids, the interferon (IFN)-γ concentration in individual cultures and the number of IFN-γ–secreting cells (ELISPOT) were also compared by one-way ANOVA followed by Tukey HSD tests. The differences were considered significant when the p value was <0.05.

RESULTS

Protective efficacy in A/Sn mice following individual or combined vaccination with plasmids harboring ts *or* asp-2 *genes*

Initially, we evaluated the protective efficacy of genetic vaccination of A/Sn mice with plasmids p154/13 (*ts* gene) and pIgSPclone9 (*asp-2* gene) administered individually or simultaneously. The first three groups of mice were immunized with four doses of plasmids pcDNA3, p154/13 or pIgSPclone9. Each dose consisted of 200 μg of DNA administered intramuscularly. A fourth group of mice was injected with four doses of a mixture containing, in each dose, 100 μg of p154/13 and 100 μg of pIgSPclone9.

After challenge with bloodstream trypomastigotes, we observed a significant reduction in peak parasitemia in animals immunized with plasmids containing *T. cruzi* genes compared to animals immunized with the control plasmid pcDNA3 (Fig. 1A, $p < 0.05$, in all cases). The values of peak parasitemia of mice immunized with either p154/13 or pIgSPclone9, or both plasmids were not statistically different ($p > 0.05$).

Dramatic differences among the groups of mice immunized

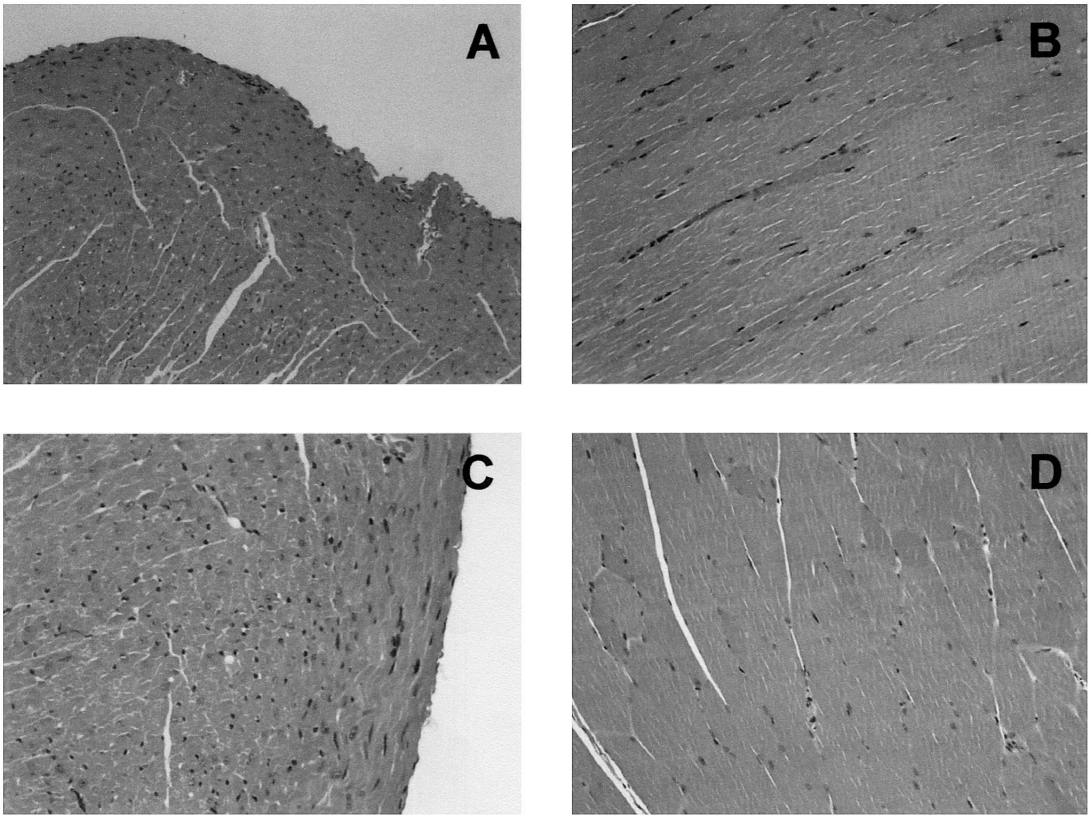

FIG. 2. Examples of histologic sections of heart and striated muscle from A/Sn mice vaccinated with plasmid DNA analyzed 100 days after infection with trypomastigotes of *Trypanosoma cruzi*. A/Sn mice were immunized as described in the legend of Figure 1 with pIgSPclone9, or the mixture p154/13 and pIgSPclone9. Three weeks after the last immunizing dose, mice were challenged with 200 *T. cruzi* trypomastigotes. After 100 days, histologic sections of heart and striated muscle were analyzed. Heart (**A**) or striated muscle (**B**) sections of a mouse immunized with pIgSPclone9. Heart (**C**) or striated muscle (**D**) sections of a mouse immunized the mixture of p154/13 and pIgSPclone9. Magnification, $\times$17. Tissue sections from mice immunized but not challenged were identical to normal nonmanipulated mice (data not shown).

with plasmids containing *T. cruzi* genes were detected when we compared the timing of mouse mortality after challenge with *T. cruzi*. Animals immunized with p154/13 survived longer than control mice ($p < 0.0001$). Nevertheless, only 3 of 22 mice (13.6%) survived. A significant delay in mouse mortality was observed in mice injected with pIgSPclone9 alone or mixed with p154/13 compared to groups of mice that received either pcDNA3 or p154/13 ($p < 0.0001$). The frequency of mouse survival in these groups was high (63.63% and 86.36%, respectively). Although a higher proportion of mice immunized with the mixture containing p154/13 and pIgSPclone9 survived infection, we were unable to detect a statistically significant difference compared to mice vaccinated with pIgSPclone9 alone ($p > 0.05$). Nevertheless, we felt that there was a tendency toward a better efficacy because the p values were close to 0.05 ($p = 0.10$, log rank test, $p = 0.08$, Pearson's χ^2).

To determine whether lower doses of plasmid could also induce protective immunity, we administered a mixture containing 20 μg (10 μg each) of p154/13 and pIgSPclone9 to each mouse. After challenge with bloodstream trypomastigotes, animals immunized with plasmids containing *T. cruzi* genes displayed a significantly lower parasitemia and survived longer than control animals. However, animals immunized with 200 μg of the mixture survived even longer, indicating that a larger dose of the mixture was more effective (data not shown).

Parasitological studies were performed in the surviving mice vaccinated with 200 μg per dose of the mixture containing p154/13 and pIgSPclone9. We found that 100% of the mice (7/7) harbored living parasites that could be isolated from their blood by hemoculture 100 days after infection. These results confirmed that protective immunity was not sterile and that vaccinated mice were unable to completely eliminate parasites. The fact that these mice still harbored parasites prompted us to evaluate the level of inflammatory response in their heart and striated muscle. In extensive histologic analysis, we were unable to find parasite nests. Also relevant was the fact that we found almost no signs of myocarditis or myositis in mice vaccinated with pIgSPclone9 or the mixture containing p154/13 and pIgSPclone9 (Fig. 2).

In vitro *correlation of protective immunity*

We then searched for *in vitro* correlation to explain the greater efficacy observed after genetic vaccination with pIgSPclone9 or the mixture containing p154/13 and pIgSPclone9. Initially, we compared the intensity of the antibody response to recombinant antigens representing TS or ASP-2. In the sera of mice immunized with p154/13, we detected high levels of antibody to recombinant TS but not to the ASP-2 recombinant protein (His-65 kDa). In the sera of mice immunized with pIgSPclone9, we detected antibodies specific for recombinant ASP-2 but not to the recombinant TS. In several independent experiments, the intensity of the antibody recognition of recombinant TS was significantly higher than the antibody recognition of ASP-2 ($p < 0.01$, Fig. 3).

Subsequently, we evaluated *in vitro* secretion of IFN-γ by the spleen cells of mice immunized with either p154/13 or pIgSPclone9 or both. Spleen cells were restimulated *in vitro* with concentrations of recombinant antigen ranging from 0.001 to 10 μg/ml. Spleen cells from mice immunized with pIgSPclone9 or the mixture and restimulated *in vitro* with doses of 0.1 or 1.0 μg/ml of recombinant ASP-2 secreted significantly higher amounts of IFN-γ than cells from mice immunized with p154/13 or the mixture and restimulated *in vitro* with the same doses of recombinant TS ($p < 0.05$ in both cases). Control mice immunized with pcDNA3 secreted little or no IFN-γ when restimulated *in vitro* with different concentrations of recombinant TS or ASP-2 (Fig. 4).

As previously reported by us, IFN-γ secreted by spleen cells after *in vitro* antigenic restimulation with recombinant proteins was dependent on the activation of CD4 T cells (Boscardin *et al*., 2003; Vasconcelos *et al*., 2003). The presence of anti-CD4

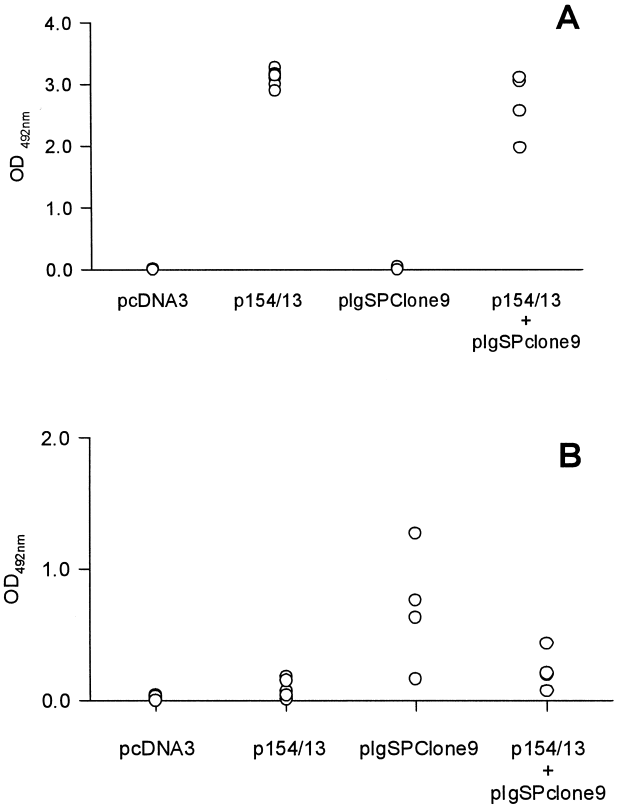

FIG. 3. Antibody immune response to *trans*-sialidase (TS) and amastigote surface protein-2 (ASP-2) in A/Sn mice immunized with either plasmid p154/13 or pIgSPclone9 or both. Mice were immunized as described in the legend of Figure 1 with pcDNA3 ($n = 6$), p154/13 ($n = 6$), pIgSPclone9 ($n = 4$), or the mixture p154/13 and pIgSPclone9 ($n = 4$). Two weeks after the last immunization, blood samples were collected and the sera assayed by enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to the recombinant *trans*-sialidase (TS; **A**) or amastigote surface protein-2 (ASP-2; **B**). Results are presented as OD_{492} values of each individual serum diluted 1000 times. Results are representative of three independent experiments. The OD_{492} values of each group were compared by one-way analysis of variance (ANOVA) and Tukey HSD tests. Higher OD_{492} values were detected in the sera of mice immunized with p154/13 or the mixture (p154/13 and pIgSPclone9) in plates coated with recombinant TS compared to the sera of mice immunized pIgSPclone9 or the mixture in plates coated with recombinant ASP-2 ($p < 0.05$ in all cases).

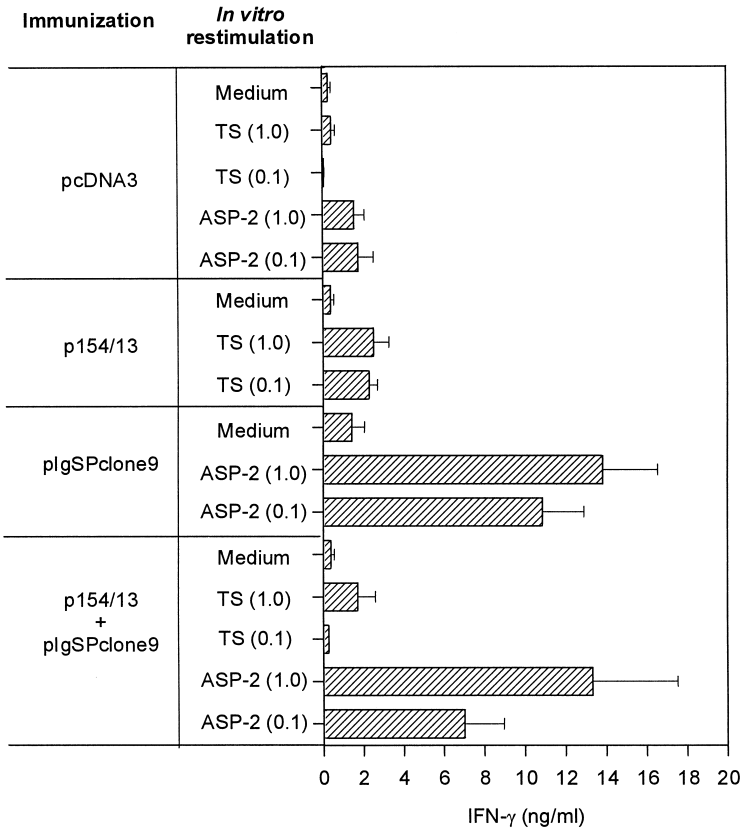

FIG. 4. Interferon (IFN)-γ secretion by spleen cells from A/Sn mice immunized with either plasmid p154/13 or pIgSPclone9 or both. Mice were immunized as described in the legend of Figure 1. Two weeks after the last immunization, spleen cells were collected from each mouse and restimulated *in vitro* with 0.1 or 1.0 μg/ml of recombinant protein. After 3 days in culture, the supernatants were collected and the concentration of IFN-γ was estimated. Results are expressed as an average of 6 individual mice $\pm$ standard deviation (SD). Results are representative of two independent experiments. The concentrations of IFN-γ were compared by one-way analysis of variance (ANOVA) and Tukey HSD tests. Higher levels of IFN-γ were detected in the supernatant of spleen cells from mice immunized with pIgSPclone9 or the mixture (p154/13 and pIgSPclone9) and restimulated *in vitro* with recombinant amastigote surface protein-2 (ASP-2) compared to cells from mice immunized with p154/13 or the mixture and restimulated *in vitro* with recombinant *trans*-sialidase (TS; $p < 0.05$ in all cases).

in culture drastically reduced the IFN-γ concentrations in these supernatants. In contrast, the presence of anti-CD8 had little or no inhibition of IFN-γ secretion by these spleen cells (data not shown).

To determine whether immunization with p154/13 or pIgSPclone9 generated specific CD8 T cells, we stimulated lymphocytes with fibroblasts (L cells) transfected with the replication-defective recombinant adenovirus expressing TS or ASP-2. The number of IFN-γ–producing cells was determined using the ELISPOT assay. We used spleen cells from mice immunized with either pcDNA3 or the mixture containing p154/13 and pIgSPclone9 as responder cells. As shown in Table 1, L cells transfected with adenovirus expressing ASP-2, stimulated IFN-γ secretion by spleen cells in individual mice immunized with p154/13 and pIgSPclone9. The IFN-γ secretion was antigen specific (i.e., dependent on ASP-2 recognition) because few cells secreting this cytokine were detected when spleen cells were stimulated with L cells transfected or not with a recombinant virus expressing TS. These IFN-γ–producing cells were induced by *in vivo* priming with plasmid pIgSPclone9 because few cells producing this cytokine were detected in the spleen of pcDNA3-immunized mice restimulated with L cells transfected with adenovirus expressing ASP-2. The lack of recognition of L cells transfected with the adenovirus expressing TS was not due to limited recombinant protein expression. The recombinant antigen could be detected easily in infected cells and in the culture supernatant (data not shown).

The phenotype of ASP-2-specific IFN-γ–producing cells was established using a selective *in vitro* depletion approach. Depletion of CD8, but not CD4 cells reduced the number of IFN-γ–producing cells to background levels (Table 1).

Contribution of CD4 and CD8 cells in protective immunity

The results described above established a correlation between the presence of specific CD4 and CD8 T cells and the protective immunity. To explore this hypothesis further, we treated DNA-vaccinated mice with anti-CD4 or anti-CD8 or both prior to the challenge with *T. cruzi*. Treatment with either mAb dramatically increased mouse susceptibility to infection compared to animals that received only rat IgG. The peak parasitemia of mice treated with anti-CD4 or anti-CD8 was significantly higher than the DNA-vaccinated mice that received only rat IgG ($p < 0.05$ in all cases; Fig. 5A).

While all of the 14 mice vaccinated with *T. cruzi* genes and treated with rat IgG survived the infection, 92.8% (13/14) of the anti-CD4–treated mice died. Likewise, 91.6% of mice vaccinated with *T. cruzi* genes and treated with anti-CD8 died after challenge. Nevertheless, a significant delay in mortality was noticed when these groups were compared to control animals injected with pcDNA3 alone ($p < 0.05$, Fig. 5B). Simultaneous treatment of vaccinated mice with anti-CD4 and anti-CD8 completely reversed protective immunity generated by plasmids containing *T. cruzi* genes. These mice displayed values of peak parasitemia higher than the other mouse groups ($p < 0.05$ in all cases, Fig. 5A). This fact is consistent with previous observation that naturally acquired immunity plays a role during acute

TABLE 1. ESTIMATION OF THE NUMBER OF IFN-γ–SECRETING SPLEEN CELLS IN A/Sn MICE IMMUNIZED WITH p154/13 AND pIgSPclone9

Experiment	*Immunization*[a]	In vitro *restimulation*[b]	In vitro *treatment*	*Number of IFN-γ–secreting cells per 0.5 × 10⁶ cells*[c]
I	pcDNA3	None	None	6.5 ± 2.1
		Adeno-TS	None	4.0 ± 0.9
		Adeno-ASP-2	None	1.6 ± 1.2
	p154/13 and pIgSPclone9	None	None	6.6 ± 2.4
		Adeno-TS	None	2.6 ± 1.3
		Adeno-ASP-2	None	59.0 ± 29.9
II	p154/13 and pIgSPclone9	None	Rat IgG	4.4 ± 1.0[d]
		Adeno-TS	Rat IgG	10.1 ± 1.0[d]
		Adeno-ASP-2	Rat IgG	59.8 ± 18.4[d]
		None	Anti-CD4	17.5 ± 4.7[d]
		Adeno-TS	Anti-CD4	12.0 ± 3.5[d]
		Adeno-ASP-2	Anti-CD4	78.1 ± 18.4[d]
		None	Anti-CD8	11.36 ± 1.7[d]
		Adeno-TS	Anti-CD8	12.1 ± 3.2[d]
		Adeno-ASP-2	Anti-CD8	12.0 ± 4.2[d]

[a]Groups of mice were immunized with a mixture of p154/13 and pIgSPclone9 or pcDNA3 alone as described in the legend of Figure 1.

[b]Two weeks after the last immunization, spleen cells were collected from each mouse and incubated with L cells transfected or not with adenovirus expressing TS (Adeno-TS) or ASP-2 (Adeno ASP-2). In experiment II, spleen cells were collected from 3 mice, pooled, and treated with rat IgG, anti-CD4, or anti-CD8 and subsequently with rabbit complement.

[c]The number of IFN-γ–secreting cells was determined using the ELISPOT assay. Results represent an average of 6 mice per group ± SD. Spleen cells from mice immunized with the mixture (p154/13 and pIgSPclone9) and restimulated *in vitro* with L cells transfected with recombinant adenovirus expressing ASP-2 had significantly higher numbers of IFN-γ–producing cells than other combinations of spleen and L cells ($p < 0.01$ in all cases, Tukey HSD). No statistically significant difference was observed when we compared the numbers of IFN-γ–producing cells among the other combinations of spleen and L cells ($p > 0.05$ in all cases).

[d]In experiment II, results are presented as the average of triplicate cultures ± SD.

TS, *trans*-sialidase; ASP-2, amastigote surface protein-2; IFN-γ, interferon-γ; IgG, immunoglobulin G; ELISPOT, enzyme-linked immunospot assay; SD, standard deviation.

T. cruzi infection. Also, these died faster than mice treated either with anti-CD4 or anti-CD8 ($p < 0.01$ in both cases, Fig. 5B).

We concluded from these experiments that, *in vivo*, both CD4 and CD8 cells are equally important for efficient reduction of peak parasitemia and improvement of mouse survival after genetic vaccination with the mixture containing p154/13 and pIgSPclone9.

DISCUSSION

Our results provide evidence that genetic vaccination of A/Sn mice with a plasmid containing the *asp-2* gene or a mixture of plasmids containing *asp-2* and *ts* genes led to a significant decrease in the peak parasitemia and an increase in mouse survival after lethal challenge with *T. cruzi* trypomastigotes. We believe that our results are extremely significant considering the high degree of susceptibility of A/Sn mice to *T. cruzi* infection. The precise nature for the high susceptibility of this strain is unknown; however, susceptibility is not linked to major histocompatibility complex (MHC) genes because B10.A mice are resistant to infection.

The fact that protective immunity was only obtained after multiple doses using large quantities of plasmids is a concern. However, we consider that this model, using a highly susceptible mouse strain will be extremely useful for future studies to determine how protective immunity can be further improved by the utilization of lower doses of antigen. For this purpose, we are currently evaluating the protective efficacy of strategies such as heterologous prime-boost using DNA for priming and adenoviral vector or recombinant protein for boosting (reviewed by Zavala *et al*., 2001; Rodrigues *et al*., 2003; Woodland, 2004). We are also developing adjuvant-loaded microspheres containing plasmid DNA. This strategy has been successfully used to lower the plasmid dosage, as well as the number of doses (Lima *et al*., 2003).

Genetic vaccination did not provide sterile immunity. Living parasites were visualized in the blood during acute phase infection and isolated 100 days after challenge from the blood of vaccinated mice. In spite of the fact that sterile immunity was not achieved, we found little or no inflammatory pathology in the heart or striated muscle of mice infected for 100 days. Our observations suggest that genetic vaccination with *asp-2* or *asp-2* and *ts* genes controlled acute phase parasitemia, survival, and the development of inflammatory responses in the

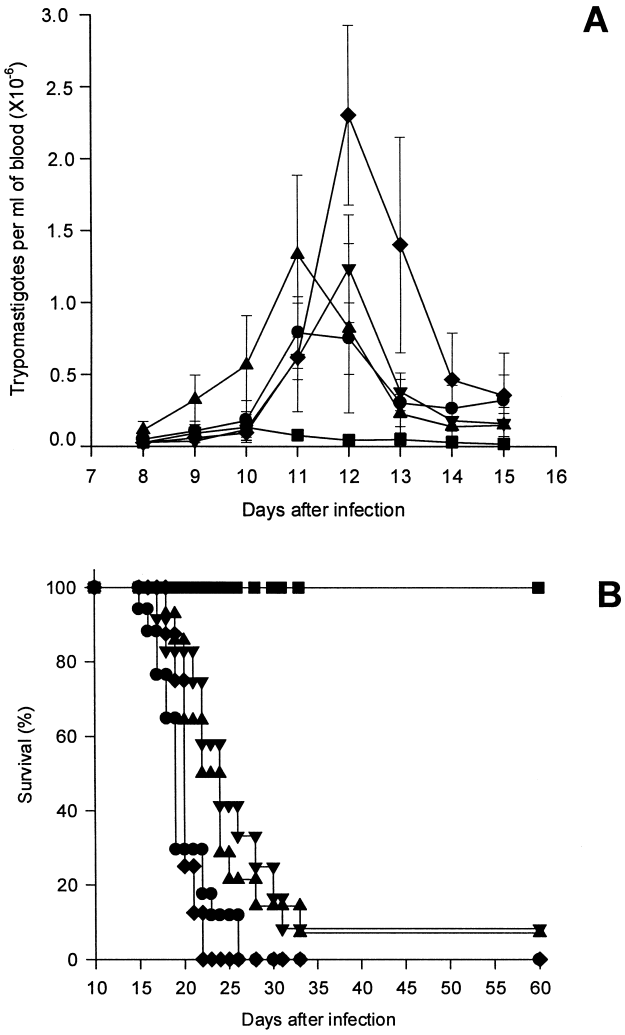

FIG. 5. Trypomastigote-induced parasitemia and mortality in mice immunized with plasmids p154/13 and pIgSPclone9 and treated with either anti-CD4, or anti-CD8 or both. Groups of mice were immunized as described in the legend of Figure 1 with the mixture of p154/13 and pIgSPclone9 or pcDNA3 alone (●). Before challenge with 200 trypomastigotes, mice immunized with the mixture of p154/13 and pIgSPclone9 were treated with rat immunoglobulin G (IgG; ■), anti-CD4 (▲), anti-CD8 (▼) or anti-CD4, and anti-CD8 (◆). **A**: The course of infection was estimated by the number of trypomastigotes per millilter of blood. The results represent the mean of 6 mice ± standard deviation (SD). The peak parasitemia of mice treated with anti-CD4 or anti-CD8 were significantly higher than the mice that received only rat IgG ($p < 0.05$ in both cases). The peak parasitemia of animals treated simultaneously with anti-CD4 and anti-CD8 was significantly higher than the parasitemia of animals treated with each antibody individually ($p < 0.05$ in both cases). Results are representative of two independent experiments. **B**: The graph shows Kaplan-Meier curves for survival. A statistically significant delay in mouse mortality was observed in mice injected with the mixture of p154/13 and pIgSPclone9 and treated with rat IgG (■, $n = 14$) compared to the other mouse groups ($p < 0.0001$ in all cases, log rank test). Mice treated simultaneously with anti-CD4 and anti-CD8 (◆, $n = 8$) died faster than mice treated with either anti-CD4 (▲, $n = 14$) or anti-CD8 (▼, $n = 12$) ($p < 0.01$ in both cases).

heart and striated muscle until 100 days after challenge. Our results confirm and extend previously described observations that DNA vaccination with *T. cruzi* genes reduce the inflammatory response in the heart and skeletal muscle of BALB/c or C57BL/6 mice when administered as prophylactic or even as therapeutic vaccines (Garg and Tarleton, 2002; Dumonteil *et al*., 2004).

The fact that mice immunized with the *asp-2* gene displayed a significantly higher degree of protective immunity when compared to animals vaccinated with the *ts* gene was initially surprising. We took advantage of our model to investigate the possible *in vitro* correlation for protective immunity. We consistently found that *in vitro* restimulation with recombinant ASP-2 induced significantly higher IFN-γ secretion than restimulation of immune cells with the same doses of recombinant TS antigen. This observation was not expected if one considers that both proteins have a similar molecular mass (approximately 65–66 kd), and genetic vaccination with the *ts* gene always generated higher titers of antibody than immunization with the *asp-2* gene (Fig. 3).

Several plausible hypotheses can be envisaged. The simplest one is the fact that ASP-2 may have a greater number of epitopes recognized by CD4 Th1 cells than TS. Nevertheless, this hypothesis alone would not fully explain the fact that, 10 to 100 times less recombinant ASP-2 was sufficient to stimulate maximal levels of IFN-γ secretion *in vitro* compared to recombinant TS. Alternatively, CD4 T-cell epitopes within ASP-2 may have a significantly higher affinity for MHC IA^k and IE^k gene products requiring lower doses of antigen for recall immune responses.

A second relevant observation made during our *in vitro* studies was the fact that immunization with the *asp-2* gene generated IFN-γ–secreting specific CD8 T cells. In contrast, immunization with the *ts* gene failed to generate TS-specific CD8 T cells. The lack of CD8 T cell epitope(s) within the TS antigen may explain why we failed to obtain protective immunity after genetic immunization with the *ts* gene even when we generated potent specific Th1 immune responses (Vasconcelos *et al*., 2003).

Experiments using *in vivo* T-cell depletion were performed and provided evidence that both subpopulations play a critical role and are equally important during protective immunity elicited by simultaneous immunization with both *T. cruzi* genes (*asp-2* and *ts*). The central participation of both CD4 and CD8 T cells was also suggested during protective immunity in BALB/c mice achieved by genetic immunization with the TS gene (Fujimura *et al*., 2001).

In summary, our results reinforce the prophylactic potential of vaccination with the *asp-2* gene by describing that significant protective immunity mediated by CD4 and CD8 cells can also be generated in a highly susceptible mouse strain, especially when administered in association with the *ts* gene.

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