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Immunization with cDNA Expressed by Amastigotes of *Trypanosoma cruzi* Elicits Protective Immune Response against Experimental Infection

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Immunization of mice with plasmids containing *Trypanosoma cruzi* genes induced specific antibodies, $CD4^+$ Th1 and $CD8^+$ Tc1 cells, and protective immunity against infection. In most cases, plasmids used for DNA vaccination contained genes encoding antigens expressed by trypomastigotes, the nonreplicative forms of the parasite. In this study, we explored the possibility of using genes expressed by amastigotes, the form of the parasite which replicates inside host cells, for experimental DNA vaccination. For that purpose, we selected a gene related to the amastigote surface protein 2 (ASP-2), an antigen recognized by antibodies and T cells from infected mice and humans, for our study. Using primers specific for the *asp-2* gene, four distinct groups of genes were amplified from cDNA from amastigotes of the Y strain of *T. cruzi*. At the nucleotide level, they shared 82.3 to 89.9% identity with the previously described *asp-2* gene. A gene named *clone 9* presented the highest degree of identity with the *asp-2* gene and was selected for immunological studies. Polyclonal antisera raised against the C terminus of the recombinant protein expressed by the *clone 9* gene reacted with an antigen of approximately 83 kDa expressed in amastigotes of *T. cruzi*. Immunization of BALB/c mice with eukaryotic expression plasmids containing the *clone 9* gene elicited specific antibodies and $CD4^+$ T-cell-dependent gamma interferon secretion. Upon challenge with trypomastigotes, mice immunized with plasmids harboring the *clone 9* gene displayed reduced parasitemia and survived lethal infection. We concluded that amastigote cDNA is an interesting source of antigens that can be used for immunological studies, as well as for vaccine development.

Trypanosoma cruzi is an obligate intracellular protozoan parasite and the etiologic agent of Chagas' disease. Despite the significant reduction in transmission observed in several countries in the last 20 years, Chagas' disease is still a major health problem for many Latin American countries, afflicting more than 15 million individuals and causing thousands of deaths every year. The poor prospect of treatment raises the possibility that immune interventions, such as immunization, could be used as an additional weapon to improve disease prevention and treatment efficacy.

Recently, independent groups established that immunization with plasmids containing *T. cruzi* genes generated immune responses mediated by antibodies, $CD4^+$ and $CD8^+$ T cells. Mice vaccinated with DNA displayed remarkable protective immunity, surviving otherwise lethal infection with *T. cruzi* (5, 9, 10, 28, 38, 45). Genes used for DNA vaccination against experimental Chagas' disease were mainly expressed by trypomastigotes of *T. cruzi*. Genes expressed by amastigotes, the form of the parasite that multiplies inside host cells, have only very recently been subject to similar studies (10). Based on the limited information on that subject, the present study was designed to explore the possibility of using a gene expressed by amastigotes for DNA vaccination against experimental Chagas' disease.

Toward that goal, to initiate our studies, we selected the gene encoding amastigote surface protein 2 (ASP-2), a surface antigen expressed by amastigotes of *T. cruzi*, that has been described previously (19, 20, 26, 47). This protein was initially described by Pan and McMahon-Pratt in 1989 (26) with the aid of a stage- and species-specific monoclonal antibody (MAb) raised against purified membrane components of *T. cruzi* amastigotes. An 83-kDa antigen was purified with the aid of the MAb, and its N-terminal amino acid sequence was determined. The observation that the purified antigen was specifically recognized by antibodies from individuals with Chagas' disease residing in geographically distant regions of South America was very important (26).

Based on the known N-terminal amino acid sequence, a cDNA corresponding to an 83-kDa surface protein was cloned by reverse transcription-PCR (RT-PCR) from RNA from amastigotes. The protein encoded by this cDNA was named ASP-2 (20). The predicted amino acid sequence encoded by the *asp-2* gene contains one aspartic acid box motif (ASP box; SXDXGXTW) present in bacterial sialidases and the highly conserved motif VTVXNVXLYNR characteristic of type three module of fibronectin. On the basis of its predicted primary structure, the *asp-2* gene was assigned to subfamily II of the sialidase/*trans*-sialidase gene superfamily of *T. cruzi* (6).

Using synthetic peptides based on the predicted amino acid sequence encoded by the *asp-2* gene, it was possible to demonstrate that ASP-2 contains multiple major histocompatibility complex (MHC) class I-restricted epitopes recognized by specific $CD8^+$ cytotoxic T lymphocytes (CTL) from mice and humans infected with *T. cruzi* (19, 47). These CTL specific for ASP-2 lysed target cells infected with *T. cruzi,* demonstrating

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that infected host cells process and present ASP-2 to CTL (19). Together with the work by Pan and McMahon-Pratt (26), these studies provided a strong immunological rationale for the selection of ASP-2 to initiate our studies on DNA immunization with genes expressed by amastigotes of *T. cruzi*.

In the present study, we characterized cDNA genes containing nucleotide sequences with high identity to the *asp-2* gene expressed by amastigotes of the Y strain of *T. cruzi*. A gene named *clone 9* shared the highest degree of identity with the previously described *asp-2* gene sequence and was selected for immunological studies of experimental genetic vaccination. BALB/c mice were immunized with eukaryotic expression plasmids harboring the *clone 9* gene. In these mice, we studied the presence of immune responses by looking at the presence of specific antibodies and gamma interferon (IFN-γ)-secreting spleen cells, as well as protective immunity against infection with bloodstream trypomastigotes.

MATERIALS AND METHODS

Mice and parasites. Female 5- to 8-week-old BALB/c and C57BL/6 mice used in this study were purchased from the University of São Paulo.

Parasites of the Y strain of *T. cruzi* were used in this study (41). Epimastigotes were maintained in liver infusion tryptose medium containing 10% (vol/vol) fetal bovine serum (FBS) (Life Technologies) at 28°C. Intracellular amastigotes were obtained from infected L6E6 cells (American Type Culture Collection, Rockville, Md.) grown in RPMI medium (Life Technologies) containing 10% FBS at 37°C in an atmosphere containing 5% CO_2. The intracellular forms were obtained from infected cells by scraping the monolayer with the aid of a cell scraper (Costar) in a buffer containing 27 mM K_2HPO_4, 8 mM Na_2HPO_4, and 26 mM KH_2PO_4, pH 7.2. The cells were lysed with the aid of a Potter-Elvehjem homogenizer. The solution containing free parasites and cell debris was placed on top of a Ficoll-Paque gradient (Pharmacia Biotech). After centrifugation at 800 × *g* for 20 min, the purified parasites were collected from the bottom of the tube. Amastigotes were subsequently washed twice in plain RPMI medium.

Trypomastigotes were collected from the extracellular medium of infected LLC-MK_2 cells (kindly provided by S. Schenkman, Universidade Federal de São Paulo-Escola Paulista de Medicina [UNIFESP], São Paulo, Brazil) 5 to 7 days after infection, centrifuged at 2,000 × *g* for 10 min, and recovered from the supernatant 3 h later.

Bloodstream trypomastigotes were obtained from mice infected 7 days earlier. Blood samples were collected from the axillary vein and transferred to a tube containing heparin. After centrifugation, the parasites were collected with plasma, centrifuged, and washed twice in phosphate-buffered saline (PBS). The number of parasites was estimated and adjusted to a concentration of 75,000 per ml. Each mouse was inoculated intraperitoneally with 0.2 ml (15,000 trypomastigotes). Parasite development was monitored by counting the number of bloodstream trypomastigotes in 5 μl of fresh blood collected from the tail vein (17). When there were more than 10^5 parasites per ml, the blood was diluted 10 times in a solution containing 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.0, to lyse red blood cells. The concentration of parasites was estimated with the aid of a hematocytometer.

RNA purification and RT-PCR. Parasite RNA was purified from the different developmental stages using TRIzol (Life Technologies) according to the manufacturer's instructions. Subsequently, the RNA was treated with RNase-free DNase I (Life Technologies) according to the protocol supplied by the manufacturer. The RNA was precipitated with ethanol, washed, and resuspended in nuclease-free water. The cDNA reaction was performed with 2.5 μg of total RNA using the ThermoScript RT-PCR system (Life Technologies). The oligo(dT) primer supplied in the kit was used during the first strand reaction, and the annealing temperature chosen was 60°C for 60 min. Further steps were followed according to the manufacturer's instructions. Control RT reactions were performed in the absence of the enzyme.

Specific oligonucleotides were designed on the basis of the previously published nucleotide sequence of the *asp-2* gene (20). All oligonucleotides used in this study were purchased from Life Technologies. The oligonucleotides and their sequences were as follows: ASP-2F forward, 5′-GGGGGTACCATGCTC TCACGTGTTGCT-3′; and ASP-GR reverse, 5′-GGGTCTAGATCAGACCA TTTTTAGTTCACC-3′ (underlined nucleotides represent *Kpn*I and *Xba*I sites, respectively). For a control, we used a pair of primers specific for the sequence of the *T. cruzi* α-tubulin gene: TUBF forward (5′-ACCGATGTTGCGGCGAT GCTTGAC-3′) and TUBR reverse (5′-CCGCGCGCTGCACCTTGGCAA-3′).

PCR was performed using the enzyme PLATINUM *Pfx* DNA polymerase (Life Technologies) according to the protocol supplied by the manufacturer. The annealing temperature used was 48°C, and the number of cycles was 35. PCR was performed in a Perkin-Elmer 2400 GeneAmp PCR system. Amplification products were electrophoresed in 1% (wt/vol) agarose gels and visualized by ethidium bromide staining and UV illumination. For DNA size markers, we used λ *Hin*dIII fragments or 100-bp DNA ladder (both purchased from Life Technologies).

Cloning and sequencing. A fragment of approximately 2.1 kb was excised from agarose gels, and the DNA was purified with Geneclean II kit (BIO 101). The purified product was cloned into the pMOSBlue vector (Amersham-Pharmacia), and after restriction analysis using restriction enzymes *Eco*RI and *Xba*I (Life Technologies), the clones containing inserts with the expected size were selected. Twenty clones were then chosen and submitted to restriction analysis using enzymes *Eco*RI, *Hin*dIII, and *Bam*HI (Life Technologies).

Automatic sequencing of double-stranded DNA was performed using the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer) on an ABI PRISM 377 sequencer (Perkin-Elmer). Initially, U19 and T7 oligonucleotides (35) present in the flanking regions of the pMOSBlue vector were used. Subsequently, to complete the sequencing of each clone, new oligonucleotides were designed to cover the entire sequence.

DNA and predicted amino acid sequences were analyzed using the DNASTAR package version 5.00 (DNASTAR, Inc.). Alignments were produced by using the Clustal W program. Analysis for potential secretory signal peptide (SP) was performed at the SignalP website (http//www.cbs.dtu.dk/services).

Pulsed-field gel electrophoresis, Northern blot analysis, and hybridization conditions. DNA was prepared from *T. cruzi* epimastigotes as described previously (23). Agarose blocks of epimastigotes of *T. cruzi* were prepared, and pulsed-field gel electrophoresis was performed at 18°C in 0.5× Tris-borate-EDTA (TBE) (2), using contour-clamped homogeneous electric field apparatus (Pharmacia Biotech). Chromosomes were fractionated on 1.2% (wt/vol) agarose gels (chromosome-grade agarose; Bio-Rad). Electrophoresis was performed as described previously (2). Gels were stained with ethidium bromide, photographed, and blotted under alkaline conditions to Hybond-N membranes (Amersham). *Hansenula wingei* DNA fragments (Bio-Rad) (that had been prepared using contour-clamped homogeneous electric field apparatus) ranging in size from 1.05 to 3.13 Mb were used as molecular size markers.

Total RNA was resuspended in RNase-free water, equilibrated in a buffer containing formamide, formaldehyde, and morpholinepropanesulfonic acid (MOPS), heated for 30 min at 70°C, and fractionated in 1% agarose formaldehyde gels (35). RNA ladder ranging from 0.24 to 9.5 kb was used as size marker (Life Technologies). Gels were photographed and transferred to membranes, and the RNA was fixed and cross-linked to Hybond-N membranes by UV irradiation.

Filters were prehybridized for 2 h at 42°C in a hybridization solution containing 50% (vol/vol) formamide (Sigma), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 10 μg of tRNA (Sigma) per ml, and 100 μg of salmon testes DNA (Sigma) per ml. Filters were hybridized overnight at the same temperature with the indicated probes labeled with [α-^{32}P]dCTP (Amersham) by using the Oligolabeling kit (Pharmacia Biotech) according to the manufacturer's instructions. The filters were washed in SSC solutions (2 to 0.1×) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) from 42 to 50°C and exposed to Hyperfilm (Amersham-Pharmacia).

Recombinant proteins based on the sequence of the *clone 9* gene: plasmid generation, *Escherichia coli* expression, and purification and production of specific antisera. For a bacterial expression vector, we used the pHIS plasmid kindly provided by Peter Sheffield (University of Virginia) (39). Two plasmids were generated based on the sequence of the *clone 9* gene: pHIS-65kDa and pHIS-25kDa. We selected these two plasmids for expression because attempts to generate a recombinant protein with the entire *clone 9* gene were not successful.

For plasmid pHIS-65kDa, the forward oligonucleotide (5′-GGG AAT TCA TGC CTC AAT GGG TC-3′) was designed to anneal with nucleotides 232 to 246 of the *clone 9* gene. For plasmid pHIS-25kDa, the forward oligonucleotide (5′-GGG GAT CCT GGT CTG GTT GGA TTC-3′) was designed to anneal with nucleotides 1474 to 1489 of the *clone 9* gene (details in Table 1) (underlined nucleotides represent *Eco*RI and *Bam*HI sites, respectively). The reverse oligonucleotide used in the PCR was ASP-GR described above. PCR was performed using the enzyme PLATINUM *Pfx* DNA polymerase, and the annealing temperature used was 48°C. The amplified PCR products were cloned into the

TABLE 1. Characteristics of the plasmids used in this study

Plasmid designation	Vector	Coding region of *clone 9* gene (bp)	No. of translated aa of *clone 9* gene	Additional sequence in the N-terminal region[a]	No. of aa in polypeptide
pHIS-25kDa	pHIS	1474–2085	492–694	MSYY**HHHHHH**DYDIPTTENLYFQGAMDPM	231
pHIS-65kDa	pHIS	232–2085	78–694	MSYY**HHHHHH**DYDIPTTENLYFQGAMDPM	645
pclone9	pcDNA3	1–2085	1–694	None	694
pSPclone9	pcDNA3	1–2085	1–694	**MKTIIALSYIFCLVFA**DYKDDDDAMDPLVTAA SVLEFCRYPSHWRPILE	742
pIgSPclone9	pcDNA3	1–2085	1–694	**MGMQVQIQSLFLLLLWVPGSRG**GT	718
pIgSP	pcDNA3	None	None	**MGMQVQIQSLFLLLLWVPGSRG**	22
pcDNA3	pcDNA3	None	None	None	0

[a] The sequences shown in boldface type are the hexahistidine tag (HHHHHH), influenza virus hemagglutinin (SP MKTIIALSYIFCLVFA), and mouse Ig κ chain SP (MQVQIQSLFLLLLWVPGSRG).

pMOSBlue vector, removed by treatment with restriction enzymes *Eco*RI and *Sal*I, and ligated into the pHIS vector treated with the same enzymes.

Plasmids pHIS-65kDa and pHIS-25kDa were transformed into *E. coli* BL-21 expression host cells (Novagen, Madison, Wis.). Protein expression was obtained by inoculation of 1 ml of a culture grown overnight in 100 ml of Luria broth containing 100 μg of ampicillin (Sigma) per ml. The culture was grown with continuous shaking at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced overnight at room temperature in the presence of 0.01 mM isopropyl-L-thio-β-D-galactoside (Life Technologies). Cells were harvested, centrifuged, and resuspended in 5 ml of a solution containing 1% (vol/vol) Triton X-100 (Sigma), 4 mg of lysozyme (Sigma) per ml, and 1 mM phenylmethylsulfonyl fluoride (Life Technologies) dissolved in PBS. The suspension was then transferred to cryogenic tubes and freeze-thawed three times in liquid nitrogen. After centrifugation at 12,500 × *g* for 20 min, the pellet was resuspended in 500 μl of a denaturing solution containing 8 M urea, 100 mM Tris-HCl (pH 9.0), 20 mM dithiothreitol (Life Technologies), and 3 mM EDTA (Sigma). After centrifugation at 12,500 × *g* for 10 min, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (35) containing 5% (vol/vol) 2-mercaptoethanol was added to 200 μl of the supernatant, and the sample was boiled for 3 min. These samples were subjected to electrophoresis on gels consisting of 10 or 12.5% (wt/vol) polyacrylamide and 0.1% (wt/vol) SDS. Gels were stained with a solution of 250 mM KCl, and the band corresponding to the recombinant protein was excised using a razor blade. The gel bands were cut in small pieces and eluted overnight in distilled water at room temperature under constant agitation. The volume was reduced with the aid of a Hetovac (Heto, Scandinavia). The purity of recombinant protein was determined by SDS-PAGE. For molecular size standards, SDS-molecular weight marker kit (Sigma) or Benchmarker Prestained Protein Ladder (Life Technologies) was used.

Before recombinant proteins were used in cell cultures, SDS was removed by dialysis against distilled water overnight at 4°C. Water was changed four times. Protein concentration was determined by gel electrophoresis using known concentrations of bovine serum albumin (BSA) (Sigma) as the standard.

C57BL/6 mice were immunized with 100 μg of the purified recombinant protein (His-25kDa) in complete Freund's adjuvant (CFA) in the hind foot. Two weeks later, mice were bled, and their sera were used for immunological assays.

Eukaryotic expression vectors: plasmid generation, purification, and immunization. Commercially available plasmid pcDNA3 (Invitrogen, San Diego, Calif.) was used as the mammalian expression vector. Plasmid pclone9 was generated by inserting the *clone 9* gene into the *Eco*RI and *Xba*I sites of plasmid pcDNA3 (details in Table 1). Plasmid pSPclone9 was generated by inserting the nucleotide sequence encoding the signal peptide of hemagglutinin protein from the influenza virus in frame with the 5′ nucleotide sequence of the *clone 9* gene into the pclone9 plasmid. The nucleotides encoding the hemagglutinin protein SP (15) were obtained from a plasmid kindly provided by Sang Won Han (UNIFESP, São Paulo, Brazil) and inserted using the *Hin*dIII and *Bam*HI sites of the pcDNA3 vector containing the *clone 9* gene. Plasmid pIgSPclone9 was obtained by inserting the nucleotide sequence encoding the mouse immunoglobulin κ chain SP (IgSP) in frame with the 5′ nucleotide sequence of the *clone 9* gene. The nucleotide sequence encoding IgSP was generated from annealing six different oligonucleotides synthesized on the basis of the sequence described in reference 14. This product was subcloned into the *Kpn*I site from pcDNA3 plasmid. The *clone 9* gene was subsequently cloned into *Kpn*I and *Xba*I sites of pcDNA3. The presence of the nucleotides encoding the hemagglutinin protein SP or IgSP was confirmed by direct sequencing of plasmids pSPclone9 and pIgSPclone9 using the T7 oligonucleotide present in the 5′-flanking region of the pcDNA3 vector. Plasmid p154/13 containing the catalytic domain of *T. cruzi trans*-sialidase used as a positive control in a few experiments was generated as described previously (5).

Plasmids were produced in *E. coli* DH5α and purified on cesium chloride density gradients according to standard protocols (35). DNA concentration was estimated to be 260 nM and confirmed by agarose gels stained with ethidium bromide. Each plasmid DNA was diluted in sterile PBS to a concentration of 1 mg/ml.

BALB/c mice were immunized according to a protocol described earlier (5). Both tibialis anterioris muscles were injected with 3.5 μg of cardiotoxin (Sigma). Five days later, 50 μg of plasmid DNA was injected intramuscularly (i.m.) at the same sites as for cardiotoxin injection (a total of 100 μg of plasmid DNA per mouse). The subsequent doses consisted of the same amount of plasmid DNA injected 3, 5, and 7 weeks after the first dose.

Ten days to five weeks after the last dose, mice were used for immunological studies or challenged with bloodstream trypomastigotes.

Immunoblotting. Parasites of the different stages of *T. cruzi* were obtained as described above. After the samples were washed in PBS twice, the parasite concentration was estimated by counting them with a hematocytometer and their concentration was adjusted to 10^9 parasites per ml. Parasite extracts were obtained by treating the parasites with a solution of PBS containing a final concentration of 1% (vol/vol) NP-40 and proteolytic enzyme inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM iodoacetamide [Sigma]). After centrifugation for 5 min at 12,500 × *g*, the supernatants were collected, mixed with SDS-PAGE sample buffer containing 2-mercaptoethanol and boiled for 3 min. The amount of liquid corresponding to 10^7 parasites was loaded onto SDS-polyacrylamide gels. After electrophoresis, the samples were transferred to nitrocellulose membranes (Millipore) for 1 h at 100 V and 4°C. The membranes were treated with PBS containing 5% (wt/vol) dry nonfat milk and 2% (wt/vol) BSA for at least 2 h and incubated with a pool of sera from mice immunized with the recombinant His-25kDa protein (diluted 1:750). After extensive washes with PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween), bound antibodies were detected by incubating the immunoblot membranes with peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Life Technologies) (diluted 1:2,000) and visualizing the chemoluminescence reaction with enhanced chemiluminescent reagent (Amersham-Pharmacia) and exposure to Hyperfilm.

ELISA and indirect immunofluorescence assay (IIA). Antibodies were detected by enzyme-linked immunosorbent assay (ELISA) using microtiter polystyrene plates (high binding; Costar) coated with the recombinant protein His-65kDa. Each well was incubated overnight at room temperature with 0.125 μg of the recombinant protein dissolved in 0.05 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.6, in a final volume of 50 μl. Unbound antigen was removed by washing the plates three times with PBS-Tween. Wells were blocked with PBS containing 5% (wt/vol) dry nonfat milk and 2% (wt/vol) BSA. After 2 h, 50-μl serum samples (diluted 800 times) from immunized and control mice were incubated for 60 min at room temperature. After five washes with PBS-Tween, wells were incubated for 60 min at room temperature with anti-mouse IgG conjugated to peroxidase (KPL) diluted 1:4,000. Bound immunocomplexes were detected with *o*-phenylenediamine (Sigma). Plates were read at OD_{492} on an ELISA reader (Labsystems Multiskan MS).

For IIA, parasites or infected cells were fixed for 30 min in PBS containing the nonpermeabilizing reagent paraformaldehyde (Merck) (3.5% [vol/vol]). After fixation, parasites were washed twice with PBS, and the solution was adjusted to a concentration of 10^7 parasites per ml. Ten-microliter portions of the parasite suspension were layered onto round, coated coverslips and allowed to stand

overnight at room temperature. Coverslips were stored at −20°C until use. Infected cells were permeabilized by treatment with PBS containing 0.2% (vol/vol) Triton X-100 for 15 min at room temperature. Blocking was performed for 30 min at room temperature in PBS containing 3% (wt/vol) BSA. Sera from mice immunized with plasmid pclone9 were diluted to 1:25 in PBS-BSA and allowed to react for 30 min at room temperature. After three washes in PBS-Tween, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1:25 was added for an additional 60 min. Coverslips were covered with 50% (vol/vol) glycerol carbonate–0.5 M bicarbonate buffer and observed using an Optishot-2 fluorescence microscope (Nikon). IIA images were acquired with a Zeiss Axiovert microscope attached to a Bio-Rad 1024UV confocal microscope using a 100× 1.4 (aperture) differential interference contrast PlanApo objective.

Cell cultures and detection of IFN-γ and NO. Cells were cultured in RPMI medium (Life Technologies) supplemented with 10 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 1% (vol/vol) nonessential amino acid solution, 1% (vol/vol) MEM vitamin solution (all purchased from Life Technologies), 100 U of penicillin and streptomycin (Sigma) per ml, and 2% (vol/vol) normal human serum. The cultures were maintained at 37°C in an atmosphere containing 5% CO_2.

Cells were obtained from the spleens of three BALB/c mice immunized with plasmid pclone9, pSPclone9, pIgSPclone9, or pcDNA3. Red blood cells were lysed in a solution of 0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.0. These cells were washed three times in plain RPMI medium and resuspended in complete medium to a concentration of 20×10^6 viable cells per ml. The assay was performed in 96-well flat-bottom plates (Costar); 0.2 ml of cell suspension was pipetted into each well, and the antigen was added at the desired concentration. Recombinant His-65kDa protein was added at final concentrations ranging from 10 to 0.1 μg/ml. Recombinant *trans*-sialidase (TS) containing the catalytic domain of *T. cruzi* TS used as a positive control in a few experiments was generated as described previously (5, 9, 30). Rat IgG and purified anti-CD4 (GK1.5) and anti-CD8 MAbs (2.43) were added to a final concentration of 10 μg/ml. We collected supernatants for cytokine determination after 5 days of culture. Each determination was done in triplicate; results are reported as means ± standard deviations (SDs).

The concentration of IFN-γ or interleukin-4 (IL-4) was estimated by capture ELISA using commercially available antibodies and recombinant cytokine (Pharmingen, San Diego, Calif.) exactly as described earlier (30). The concentration of cytokine in each sample was determined from standard curves executed in parallel with known concentrations of recombinant IFN-γ or IL-4. The detection limit of the assays was 0.2 ng/ml.

Nitrite production by cultured cells was assessed by incubation of 50 μl of each supernatant with 50 μl of Griess solution (1% [wt/vol] sulfanilamide, 0.1% [wt/vol] naphthylene diamine dihydrochloride, 2% [vol/vol] H_3PO_4; all purchased from Sigma). The absorbance was measured at OD_{540} on an ELISA reader. Known concentrations of sodium nitrite diluted in culture medium were used as standards.

ELISPOT assay for the detection of peptide-specific IFN-γ-producing cells. The enzyme-linked immunospot (ELISPOT) assay was performed essentially as described earlier (25, 29). Briefly, the plates were prepared by coating the wells of 96-well Multiscreen HA nitrocellulose plates (Millipore) with a solution of sterile PBS containing 10 μg of the anti-mouse IFN-γ MAb R4-6A2 (Pharmingen) per ml (60 μl of the PBS-MAb solution was used to coat each well). After overnight incubation at room temperature, the MAb solution was removed by sterile aspiration and the plates were washed three times with plain RPMI medium under sterile conditions. Plates were blocked by incubating wells with 100 μl of RPMI medium containing 10% (vol/vol) FBS for at least 2 h at 37°C.

Responder cells were obtained from the spleens of three BALB/c mice immunized four times with plasmid pclone9, pIgSPclone9, or pcDNA3. For a positive control, we used spleen cells from three BALB/c mice immunized with plasmid p154/13. Responder cells were washed three times in plain RPMI medium and resuspended in complete RPMI medium to a concentration of 5×10^6 viable cells per ml. Complete RPMI medium was produced as described above except that it contained 10% (vol/vol) FBS (HyClone, Logan, Utah), and we added recombinant human IL-2 (kindly provided by Hoffman-LaRoche) (30 U/ml) to the medium.

Antigen-presenting cells were prepared by irradiating A20J cells (kindly provided by Moriya Tsuji, New York University School of Medicine) for 45 min. These cells express MHC class I and II molecules that improve peptide presentation to specific T cells. Cells were then washed three times in plain RPMI medium. Their viability was determined by trypan blue dye exclusion, and their concentration was adjusted to 10^6 cells/ml in complete RPMI medium. Some cells were incubated with the indicated synthetic peptide at a final concentration of 4 μM for 30 min at 37°C.

One hundred microliters of a suspension containing responders or antigen-presenting cells was pipetted into each well. The plates were incubated (without shaking) for 24 h at 37°C in an atmosphere containing 5% CO_2.

After incubation, the majority of the cultured cells were flicked off the plates. To remove residual cells, plates were washed three times with PBS and three times with PBS-Tween. Each well received 75 μl of biotinylated anti-mouse IFN-γ MAb XMG1.2 (Pharmingen) diluted in PBS-Tween to a final concentration of 2 μg/ml. Plates were incubated overnight at 4°C. Unbound antibodies were removed by washing the plates at least six times with PBS-Tween. Peroxidase-labeled streptavidin (KPL) was added at a 1:800 dilution in PBS-Tween in a final volume of 100 μl/well. Plates were incubated for 1 to 2 h at room temperature and then washed three to five times with PBS-Tween and three times with PBS.

The reactions on the plates were developed by adding peroxidase substrate (50 mM Tris-HCl [pH 7.5] containing 3,3′-diaminobenzidine tetrahydrochloride [1 mg/ml] and 30% hydrogen peroxide solution [1 μl/ml] [both from Sigma]) (100 μl/well). After incubation at room temperature for 15 min, the reaction was stopped by discarding the substrate solution and rinsing the plates under running tap water. Plates were dried at room temperature, and spots were counted with the aid of a stereomicroscope (Nikon).

Synthetic peptides. Synthetic peptides based on the predicted amino acid sequence of the *clone 9* gene were purchased from New England Peptides (Fitchburg, Mass.). As estimated by high-pressure liquid chromatography analysis, the purity of the synthetic peptides varied from 78 to 99%. Synthetic peptide $TS_{359\text{-}367}$ (IYNVGQVSI) was purchased from Neosystem (Strasbourg, France). As estimated by high-pressure liquid chromatography analysis, $TS_{359\text{-}367}$ was more than 90% pure.

Statistical analysis. The Student *t* test was used to compare the possible differences in the mean values of IFN-γ and nitrite concentrations. The peak parasite loads observed in the different mouse groups were compared by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests. The Mann-Whitney test was used to compare the OD_{492}s obtained by ELISA using sera from mice immunized with the different plasmids. Log-rank test was used to compare mouse survival times after challenge with *T. cruzi*. The differences were considered significant when the *P* value was <0.05.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in GenBank with accession numbers AY186572 (*clone 9*), AY186574 (*clone 13*), AY186575 (*clone 105*), and AY186576 (*clone 117*).

RESULTS

Identification and characterization of genes related to *asp-2* present in the cDNA of amastigotes of the Y strain of *T. cruzi*. In an attempt to characterize genes expressed by the Y strain of *T. cruzi* related to the previously described *asp-2* gene, RT-PCR was performed using an oligo(dT) primer, a pair of primers based on the published sequence of *asp-2*, and RNA extracted from the different developmental stages of the parasite. A single band of ~2.1 kb was obtained by RT-PCR using RNA from amastigotes or epimastigotes (Fig. 1A). PCR products were not observed in the absence of reverse transcriptase.

In contrast, in several independent experiments, specific PCR products were not detected when RT-PCR was performed using trypomastigote RNA. This absence cannot be attributed to the trypomastigote RNA itself, because specific PCR products could be readily obtained in the presence of primers specific for the α-tubulin gene of *T. cruzi* (Fig. 1B).

DNA products obtained from PCR using cDNA from amastigotes were cloned into the pMOSBlue vector. Twenty clones were subjected to restriction analysis, and four different restriction patterns (named 1 through 4) were observed. Clones belonging to restriction pattern 1 or 2 were more common in the cDNA representing 9 or 5 of the 20 clones studied, respectively. Restriction patterns 3 and 4 were less represented in the amastigote cDNA (each corresponding to 3 of the 20 clones). Similar proportions were observed when we studied clones

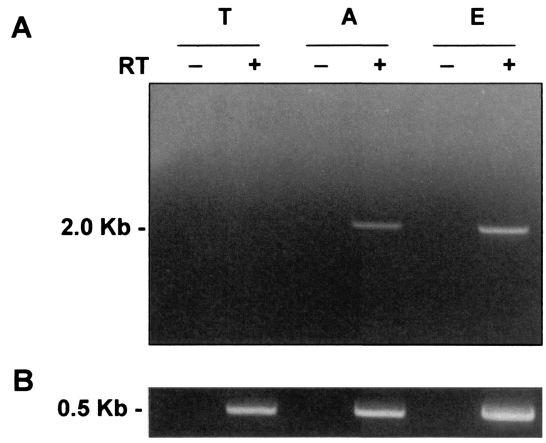

FIG. 1. RT-PCR using RNA purified from different developmental stages of the Y strain of *T. cruzi* and oligonucleotide primers based on the previously described *asp-2* gene sequence. RT-PCR was performed using oligo(dT) primer and total RNA purified from trypomastigotes (T), amastigotes (A), and epimastigotes (E) of *T. cruzi* (Y strain) in the presence (+) or absence (−) of the reverse transcriptase (RT) enzyme. PCR was performed using a pair of primers based on the previously described sequence of the *asp-2* gene (A) or *T. cruzi* α-tubulin gene (B).

isolated from different RT-PCR mixtures. The observation made when we performed PCR using an oligonucleotide representing the splice leader sequence of *T. cruzi* as the forward primer was also important. Using this oligonucleotide as a primer, restriction pattern 1 or 2 represented 12 or 6 of 18 clones studied, respectively. Clones representing restriction pattern 3 or 4 were not observed in this case (data not shown). We consider this result important, because the splice leader sequence is present only in mature mRNA of *T. cruzi*.

DNA sequences were obtained from two clones representing each restriction pattern. Comparison of the DNA sequences of the two clones representing each pattern showed minor but significant differences, indicating that they were distinct genes (data not shown). We selected *clones 9*, *13*, *117,* and *105* to represent restriction patterns 1, 2, 3, and 4, respectively. The *clone 9* gene shared the highest degree of identity with the *asp-2* gene (89.9 or 82.7% identity at the nucleotide or predicted amino acid sequence levels, respectively). The other genes revealed decreasing degrees of identity with the *asp-2* gene sequence.

At the nucleotide level, *clone 117* (pattern 3) and *clone 105* (pattern 4) presented a high degree of identity when compared to each other (98.4%). The *clone 117* and *clone 105* genes had stop codons at nucleotides 309 and 285, respectively. Despite the identity with *asp-2*, all genes isolated from amastigote cDNA with restriction pattern 3 or 4 had stop codons in their 5′ region. These genes most likely represent pseudogenes which are transcribed but not translated in *T. cruzi* (1). Figure 2 shows a Clustal W multiple-sequence alignment of the predicted amino acid sequences of *clones 9*, *13*, *117*, *105,* and the previously described *asp-2* gene. The predicted amino acid sequences of *clone 9* or *clone 13* contain a sequence encoding a highly probable SP sequence at the N terminus that contains a strongly hydrophobic, noncharged core represented by amino acids (aa) 39 to 66. The predicted cleavage site of SP is between aa 66 to 67 (Fig. 2).

The predicted amino acid sequence of the *clone 13* or *clone 9* gene contains, respectively, one or two ASP box motifs (SXDXGXTW) present in bacterial sialidases. Both genes contain the sequence encoding the highly conserved motif VTVXNVXLYNR characteristic of type three module of fibronectin. These sequences are common to members of the sialidases/*trans*-sialidase gene superfamily of *T. cruzi* (6).

Because the *clone 9* gene shared the highest identity with the *asp-2* gene sequence, it was selected for subsequent studies. The genomic distribution of the *clone 9* gene was determined by chromosome mapping, and this gene hybridized with many chromosomal bands of the Y strain genome (Fig. 3A, lane 2). Similar results were observed when we used the *clone 13* gene (data not shown). In contrast, the parasite α-tubulin gene used as a control hybridized only with two bands of the Y strain genome (Fig. 3A, lane 3). This result indicates that the *clone 9* and *clone 13* genes belong to a relatively large family of genes distributed in different chromosomes of the parasite.

By Northern blotting, the *clone 9* gene hybridized with two RNA populations of different sizes present in the different developmental stages of the parasite. The shorter RNA population was detected in RNA isolated from amastigotes or epimastigotes. The longer RNA population was detected in trypomastigotes or epimastigotes (Fig. 3B). Identical results were obtained when the *clone 13* gene was hybridized with RNA purified from the different developmental stages of the parasite (data not shown).

Antisera raised against the C terminus of the recombinant protein encoded by the *clone 9* gene recognizes an ~83-kDa protein in amastigotes of *T. cruzi*. To verify whether the *clone 9* gene did in fact encode a protein with characteristics similar to those of ASP-2, a pHIS protein expression construct was designed on the basis of the *clone 9* sequence for the production of a fusion protein, development of specific polyclonal antisera, and immunoblot analysis. The pHIS construct His-25kDa (Table 1) represented the C terminus of the recombinant protein encoded by the *clone 9* gene. We selected this region of the protein, because its predicted amino acid sequence shared a high degree of identity (180 of 203 aa) with the same region of ASP-2 (Fig. 2).

Sera from mice immunized with the fusion protein were employed to develop immunoblots containing extracts of trypomastigotes, amastigotes, and epimastigotes, as well as the recombinant protein. As shown in Fig. 4, the recombinant protein His-25kDa (lane 5) and a single band of ~83 kDa present in extracts of amastigotes of *T. cruzi* (lane 7) were recognized. Two smaller proteins of ~67 and ~47 kDa were recognized in extracts of trypomastigotes and epimastigotes, respectively (lanes 6 and 8). Antibody recognition was specific, because sera from mice immunized with CFA alone failed to react with any protein (lanes 1 to 4).

Presence of specific antibodies in the sera of mice immunized with eukaryotic expression vectors containing the *clone 9* gene. In order to study the immune response induced by DNA immunization with different eukaryotic expression plasmids, we used three different plasmids containing the *clone 9* gene. These three plasmids differ in the presence in the 5′ region of the *clone 9* gene of a DNA sequence encoding a secretory

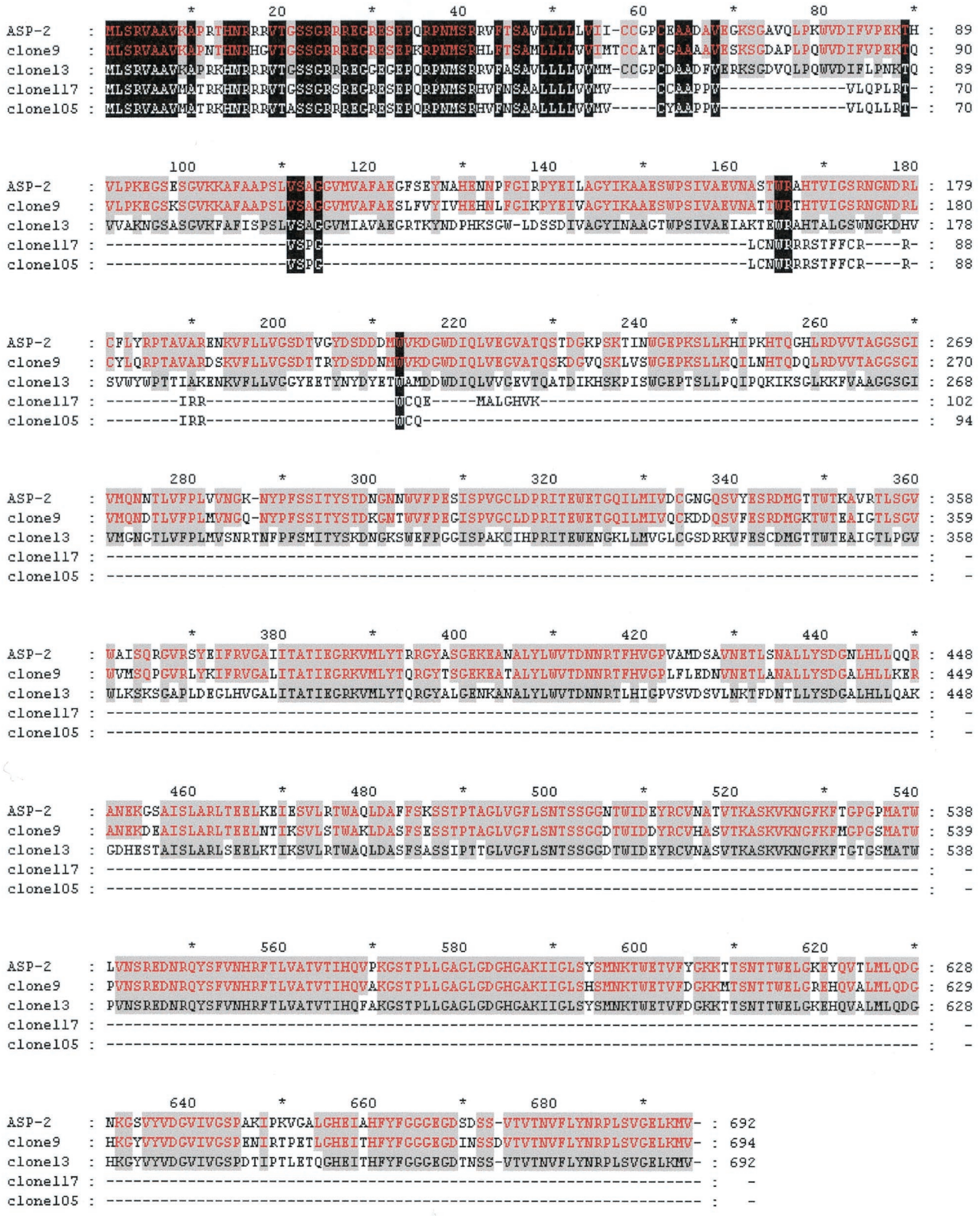

FIG. 2. Clustal W multiple-sequence alignment of the predicted amino acid sequences of *clone 9*, *13*, *117,* and *105* genes isolated from amastigote cDNA of the Y strain of *T. cruzi* and the previously described *asp-2* gene. The predicted amino acid sequences of *clone 9* (pattern 1), *clone 13* (pattern 2), *clone 117* (pattern 3), and *clone 105* (pattern 4) are shown. A black background indicates 100% identity in the predicted amino acid sequences among all clones analyzed. A gray background indicates 100% identity in the predicted amino acid sequences among the *asp-2*, *clone 9,* and *clone 13* genes. Red letters indicate 100% identity in the predicted amino acid sequences for the *clone 9* and *asp-2* genes. Dashes represent gaps introduced for optimal alignment.

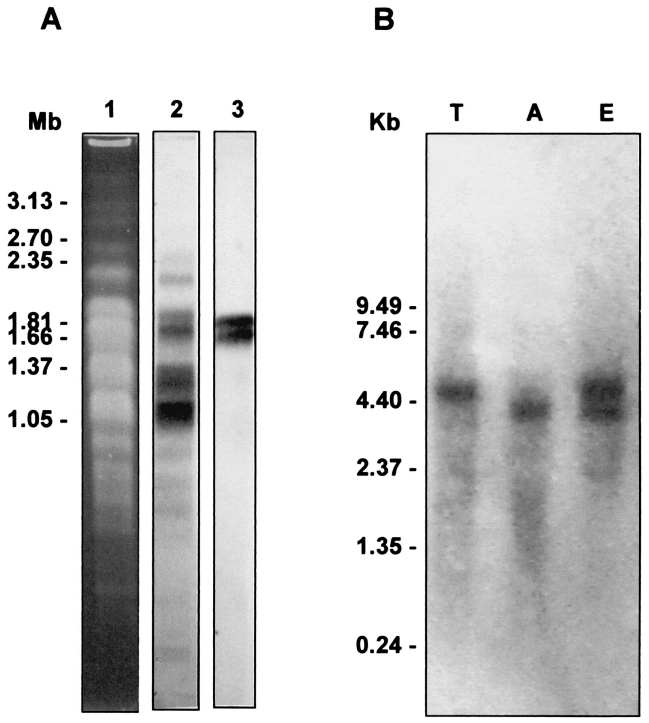

FIG. 3. Chromosome location and Northern blot analysis using the *clone 9* gene as a probe. (A) Chromosome mapping of the *clone 9* gene was performed by pulsed-field gel electrophoresis using DNA from epimastigotes. Chromosomes were stained with ethidium bromide (lane 1) and blotted onto nylon membranes. Membranes were hybridized with the radioactively labeled *clone 9* gene (lane 2) or with *T. cruzi* α-tubulin gene (lane 3). (B) Northern blot analysis was performed using total RNA purified from trypomastigotes (T), amastigotes (A), or epimastigotes (E) of *T. cruzi* (Y strain).

SP. While pclone9 contained only the *clone 9* gene, plasmid pSPclone9 or pIgSPclone9 also contained DNA sequences encoding the secretory SP of the influenza virus hemagglutinin or mouse Ig κ light chain, respectively (Table 1).

BALB/c mice were immunized i.m. with plasmid pclone9, pSPclone9, or pIgSPclone9. Control mice were immunized with pcDNA3 or pIgSP. The presence of specific antibodies was determined by ELISA using the recombinant protein His-65kDa as an antigen (Table 1). Specific antibodies were detected in most of the animals immunized with each of the three plasmids containing the *clone 9* gene (Fig. 5).

By IIA, it was possible to determine that intra- and extracellular amastigotes, but not extracellular trypomastigotes, of *T. cruzi* were specifically recognized by antibodies from mice immunized with plasmid pclone9 (Fig. 6B and D). IIA results were negative when we used sera from mice immunized with control plasmid pcDNA3 (data not shown).

Cellular immune responses of mice immunized with eukaryotic expression plasmids containing the *clone 9* gene. Next, we assessed whether immunization with eukaryotic expression plasmids containing the *clone 9* gene elicited a cell-mediated immune response, as estimated in vitro by antigen-specific IFN-γ or IL-4 secretion. Upon in vitro stimulation with the recombinant protein His-65kDa but not TS, spleen cells from mice immunized with plasmid pclone9, pSPclone9, or pIgSPclone9 secreted IFN-γ. This response was dependent on in vivo priming, because spleen cells from mice injected with control plasmid pcDNA3 failed to secrete IFN-γ upon stimulation with the recombinant protein (Table 2). These supernatants were also tested for the presence of the type 2 cytokine IL-4. On repeated occasions, we were unable to detect it (data not shown).

Secretion of IFN-γ was dependent on the activation of $CD4^+$ T cells. When compared to culture containing rat IgG, the presence of an anti-CD4 MAb significantly inhibited the antigen-specific IFN-γ production by spleen cells of mice immunized with plasmid pclone9, pSPclone9, or pIgSPclone9 ($P < 0.01$ in all cases) (Table 2). In contrast, the addition of an anti-CD8 MAb to the cultures did not cause a significant reduction in the IFN-γ secretion by these spleen cells ($P > 0.05$ in all cases) (Table 2). The addition of both MAbs did not further increase the inhibition observed in the presence of an anti-CD4 MAb (Table 2).

In addition to IFN-γ production, we measured the presence of NO in the supernatants of spleen cell cultures. Upon in vitro stimulation with the recombinant protein His-65kDa, a significant increase in NO concentration was observed in the supernatants of spleen cells from mice immunized with plasmids containing the *clone 9* gene ($P < 0.05$) (Table 3). In contrast, NO concentrations in the supernatants of spleen cells from mice immunized with the control plasmid pcDNA3 cultured in the presence or absence of the recombinant protein His-65kDa were not statistically different ($P > 0.05$) (Table 3).

The increase in the NO concentration in the supernatant of spleen cells stimulated by the recombinant protein His-65kDa was dependent on the activation of $CD4^+$ T cells. As found for IFN-γ secretion, the addition of an anti-CD4 MAb significantly reduced the NO accumulation in the supernatant of cultures containing spleen cells from mice immunized with plasmid pclone9 (Table 4).

In order to determine whether we could detect specific

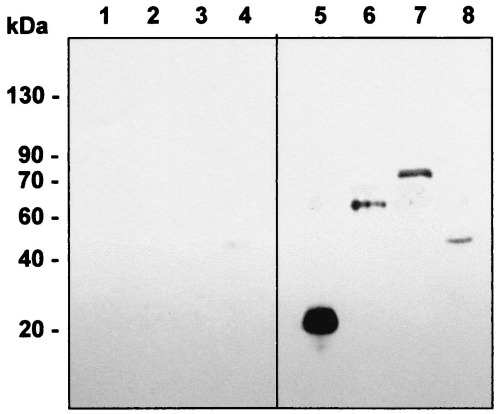

FIG. 4. Immunoblot using sera collected from mice immunized with the purified recombinant protein His-25kDa. Purified recombinant protein His-25kDa (0.3 μg) or parasite extracts (equivalent to 10^7 parasites) were added to each lane of a SDS-polyacrylamide gel (reducing conditions). Immunoblot strips contained the recombinant protein His-25kDa (lanes 1 and 5) or extracts of trypomastigotes (lanes 2 and 6), amastigotes (lanes 3 and 7), or epimastigotes (lanes 4 and 8). These strips were incubated with a pool of sera from mice immunized with CFA alone (lanes 1 to 4) or CFA plus purified recombinant protein His-25kDa (lanes 5 to 8).

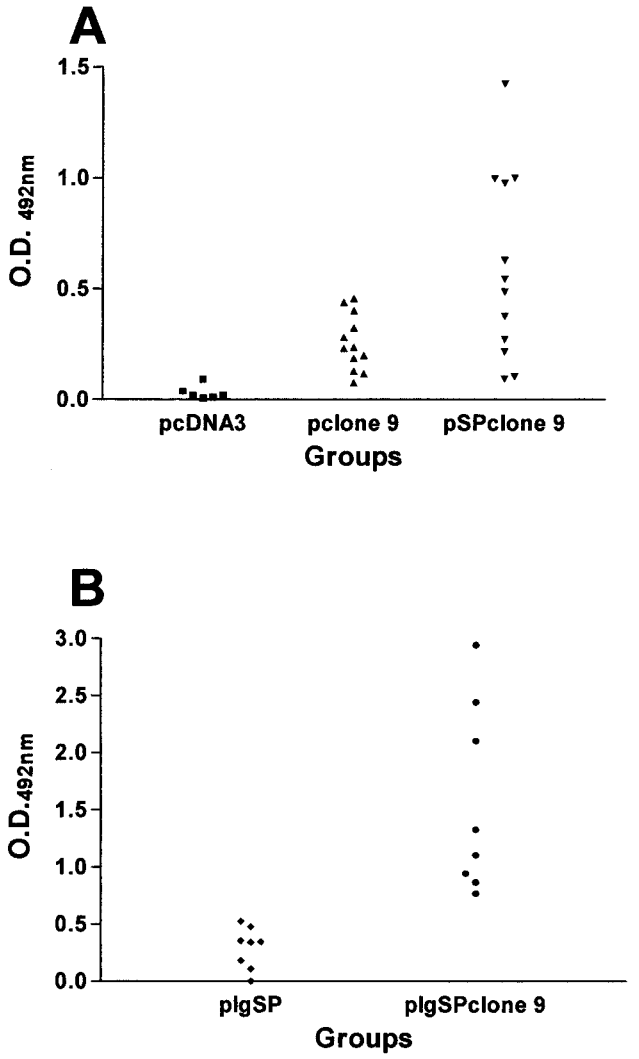

FIG. 5. Antibody immune response of mice immunized with eukaryotic expression plasmids containing the *clone 9* gene. BALB/c mice were immunized with plasmids containing the *clone 9* gene (pclone9, pSPclone9, and pIgSPclone9) or control (empty) plasmids (pcDNA3 or pIgSP). Two weeks after the last immunization, blood samples were collected, and the sera were assayed by ELISA for the presence of antibodies to the recombinant protein His-65kDa. Results are presented as the OD_{492}s of each individual serum sample diluted 800 times. The OD_{492} values of the different groups were compared by the Mann-Whitney test. The *P* values for the OD_{492} values of the different groups were as follows: $P = 0.001$ for pclone9 versus pcDNA3, $P < 0.001$ for pSPclone9 versus pcDNA3, $P = 0.0466$ for pclone9 versus pSPclone9, and $P < 0.001$ for pIgSPclone9 versus pIgSP.

$CD8^+$ T cells in the spleens of mice immunized with plasmid pclone9 or pIgSPclone9, we synthesized 14 peptides based on the predicted amino acid sequence of the *clone 9* gene. Peptides were selected by the presence of putative sites for binding to the MHC class I H-$2K^d$ or H-$2L^d$ gene product of BALB/c mice (4, 7). Peptides with putative sites for binding to H-$2D^d$ were not present in the predicted amino acid sequence of the *clone 9* gene (3).

Spleen cells from mice immunized with DNA were tested by the ELISPOT assay for their ability to secrete IFN-γ upon in vitro stimulation with target A20J cells coated with each of these peptides. This assay was selected because it is very sensitive and specific (25, 29). In repeated experiments, we could not detect the presence of peptide-specific IFN-γ-secreting cells in the spleens of mice immunized with plasmid pclone9 or pIgSPclone9 (Table 5). This failure could not be attributed to the ELISPOT assay itself or to the responder cells, because IFN-γ secretion could be detected when we used the recombinant protein His-65kDa as the antigen (Table 5). Also, we detected the presence of IFN-γ-secreting cells in the spleens of mice immunized with plasmid p154/13 which contains the gene encoding the catalytic domain of *T. cruzi* TS. These cells were specific for the $TS_{359-367}$ peptide (IYNVGQVSI) described as a $CD8^+$ T-cell epitope in our earlier study (30).

Protective immunity in mice immunized with plasmids containing the *clone 9* gene. In order to determine whether DNA immunization could induce protective immunity against *T. cruzi* infection, BALB/c mice immunized with plasmid pclone9, pSPclone9, or pcDNA3 were challenged with bloodstream trypomastigotes. In mice immunized with plasmid pclone9 or pSPclone9, we observed a significant reduction in the peak parasite level compared to those in animals immunized with pcDNA3 plasmid ($P < 0.0001$ by one-way ANOVA) (Fig. 7A). Most importantly, none of the mice immunized with plasmid pclone9 or pSPclone9 died (Fig. 7B). While 100% of the animals immunized with plasmid pclone9 or pSPclone9 survived the infection, 90% of mice injected with plasmid pcDNA3 died. This experiment was reproduced with identical results. In both experiments, a total of 36 BALB/c mice were immunized with plasmid pclone9 or pSPclone9. All animals survived the infection. In contrast, 16 of the 17 mice immunized with pcDNA3 died ($P < 0.0001$ by log-rank test).

DISCUSSION

The aim of our study was to explore the possibility of using genes expressed by amastigotes, the intracellular replicating forms of *T. cruzi*, for experimental DNA vaccination. For that purpose, we selected for our study the gene encoding the ASP-2 protein, an antigen recognized by antibodies, as well as potentially protective immune T cells from mice and humans infected with *T. cruzi* (19, 26, 47).

In a detailed characterization of the *asp-2* cDNA expressed by amastigotes of *T. cruzi* (Y strain), we isolated cDNA genes displaying four distinct restriction patterns. Each restriction pattern may include two or more distinct genes. Sequence analysis of these genes revealed 82.3 to 89.9% identity at the nucleotide level with the previously described *asp-2* gene isolated from the cDNA of the Brazil strain of *T. cruzi*. On the basis of their high degree of identity with the *asp-2* gene, they can be assigned as new members of subfamily II of the sialidase/*trans*-sialidase gene superfamily of *T. cruzi*. Southern blot hybridization using *clone 9* and *clone 13* genes confirmed that they belong to a family of genes distributed in multiple chromosomes of the Y strain genome. The isolation of these cDNA clones defined a new group of genes similar to *asp-2* expressed in the replicating forms of *T. cruzi*.

Members of subfamily II have been shown to be expressed in both trypomastigotes and amastigotes of *T. cruzi* (6, 8, 16, 20, 27, 33, 34, 36). RT-PCR analyses using specific primers showed

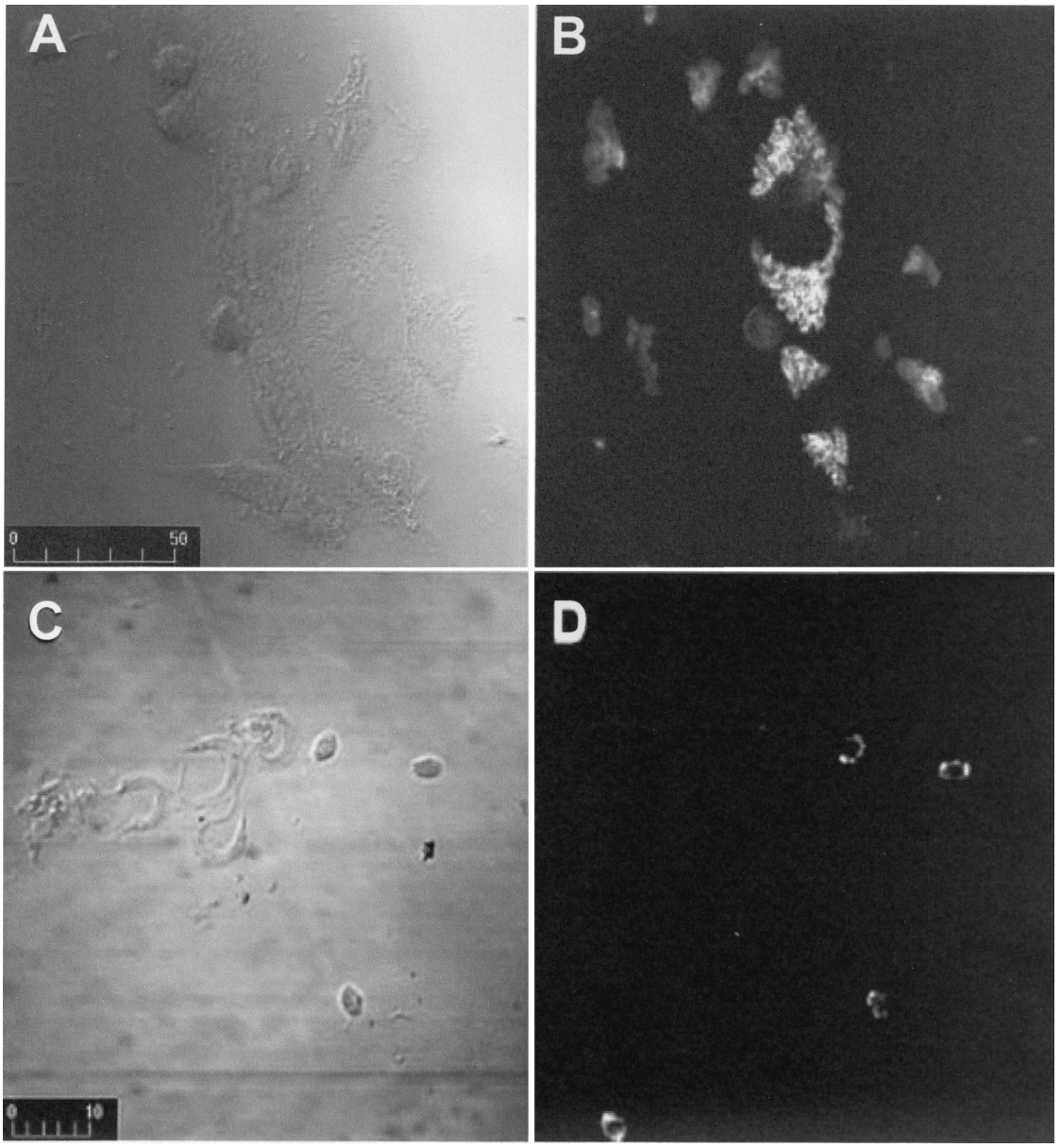

FIG. 6. IIA recognition of amastigotes, but not of trypomastigotes, of *T. cruzi* by immune sera from mice immunized with a plasmid containing the *clone 9* gene. Intracellular amastigotes (A and B) or mixtures of extracellular amastigotes and trypomastigotes from cultures (C and D) were analyzed by IIA. Parasites were incubated with a pool of sera derived from mice immunized with plasmid pclone9 and imaged under Nomarski differential interference contrast (A and C) or fluorescence (B and D) microscopy. Bars, 10 and 50 μm.

that the presence of genes related to *asp-2* in the Y strain of *T. cruzi* is restricted to the cDNA of amastigotes and epimastigotes (Fig. 1A). In contrast, Northern blot analysis reproducibly revealed the presence of mRNA that hybridizes with *clone 9* and *clone 13* genes in the different developmental stages of the parasite, including trypomastigotes. Unlike amastigotes, trypomastigotes had a longer form of RNA not seen in the intracellular stages (Fig. 3B). These contrasting results can be explained by the fact that PCR was performed with cDNA generated with an oligo(dT) primer. It is possible that Northern blot analysis detected trypomastigote mRNA which does not contain a poly(A) tail. In agreement with this possibility is the fact that specific amplification products were obtained when PCR was performed with trypomastigote cDNA generated by RT in the presence of the reverse 3′ oligonucleotide ASP-GR (data not shown).

Alternatively, it is possible that *clone 9* and *clone 13* genes hybridized with mRNA from other members of subfamily II expressed in trypomastigotes of *T. cruzi*. Indeed, comparison of *clone 9* nucleotide sequence with other genes available in GenBank revealed 62 to 63.6% identities with several genes present in trypomastigote cDNA (GenBank accession numbers L31947/Z32744, M58466, U02613, U02615, and L13844 [8, 33, 34]).

The fact that we found four distinct groups of genes in PCR products of cDNA from amastigotes raised the question of

TABLE 2. IFN-γ secretion by spleen cells from BALB/c mice immunized with different plasmids upon in vitro restimulation with the recombinant protein His-65kDa

Plasmid[a]	IFN-γ concn (ng/ml)[b]						
	No antigen	Rec. TS	Rec. His-65kDa				
			No Ab	Rat IgG	Anti-CD4 MAb	Anti-CD8 MAb	Anti-CD4 and anti-CD8 MAbs
pcDNA3	<0.2	0.27 ± 0.01	<0.2	<0.2	<0.2	<0.2	<0.2
pclone9	<0.2	<0.2	5.82 ± 0.28[c]	5.28 ± 1.55	0.84 ± 0.29[d]	3.91 ± 0.59[e]	1.12 ± 0.59[d]
pSPclone9	<0.2	NT[f]	4.16 ± 1.34[c]	4.79 ± 0.95	0.89 ± 0.21[d]	3.24 ± 0.50[e]	0.56 ± 0.07[d]
pIgSPclone9	<0.2	0.43 ± 0.13	3.29 ± 1.19[c]	3.45 ± 0.54	0.65 ± 0.07[d]	2.91 ± 0.33[e]	0.52 ± 0.07[d]

[a] BALB/c mice were immunized with the indicated plasmid as described in Materials and Methods. Two weeks after the last immunization, pooled spleen cells were collected from three mice.
[b] Spleen cells were stimulated in vitro with 10 μg of recombinant (Rec.) protein His-65kDa or recombinant TS per ml in the presence or absence of 10 μg of rat IgG per ml or with anti-CD4 or anti-CD8 MAb or anti-CD4 and anti-CD8 MAbs. The concentration of IFN-γ was estimated in cell culture supernatants, and results are expressed as averages of triplicate cultures ± SDs. This experiment was reproduced three times with identical results. No Ab, no antibody.
[c] Significantly different from values for the supernatants of cells cultured in the absence of the recombinant protein His-65kDa or in the presence of recombinant TS ($P < 0.01$ by the Student *t* test).
[d] Significantly different from values in the supernatants of cells cultured in the presence of rat IgG ($P < 0.01$ by the Student *t* test).
[e] Not significantly different from values in the supernatants of cells cultured in the presence of rat IgG ($P > 0.05$ by the Student *t* test).
[f] NT, not tested.

which one actually encodes the Y strain ASP-2. Clones representing restriction pattern 1, for example, the *clone 9* gene, represented the majority of clones obtained by RT-PCR using RNA from amastigotes. Also, at the level of predicted amino acid sequences, the *clone 9* gene exhibited the highest identity with the *asp-2* gene (82.7%). The second group of genes most commonly isolated from amastigote cDNA was represented by the *clone 13* gene. At the level of predicted amino acid sequence, this gene displayed lower degrees of identity with the *asp-2* and *clone 9* genes (72 and 71.1%, respectively).

The above observations led us to select the *clone 9* gene as the most likely candidate for the gene encoding ASP-2 of *T. cruzi* (Y strain). In support of this hypothesis, we found that antibodies prepared against a recombinant protein based on the C terminus of the protein encoded by the *clone 9* gene recognized a band of ~83 kDa in amastigote extracts on an immunoblot. This result lends further support that the *clone 9* gene does indeed encode at least part of the ASP-2 antigen expressed by amastigotes of the Y strain of *T. cruzi*.

Although it is clear that an 83-kDa protein related to the *clone 9* gene is expressed in amastigotes of *T. cruzi*, it is still not clear whether epimastigotes and trypomastigotes express this antigen. The lack of recognition by specific antibodies of an 83-kDa molecule can be explained by posttranslational modifications. A ~47-kDa band was recognized in epimastigotes by antibodies specific for the His-25kDa protein. This protein had been recognized previously using polyclonal antibodies specific for a recombinant protein encoded by the *asp-2* gene of the Brazil strain of *T. cruzi* (20). We also detected a cross-reaction with a ~67-kDa polypeptide present in trypomastigotes. The nature of the identity of these polypeptides with ASP-2 antigen is unknown and remains to be investigated.

The fact that at the level of the predicted amino acid sequences, the *clone 9* gene shared 82.7% identity with the *asp-2* gene isolated from the *T. cruzi* Brazil strain is noteworthy. Sequence conservation was observed in several of the previously described epitopes recognized by mouse and human $CD8^+$ T cells primed during infection. For example, the sequences encoding two epitopes recognized by H-$2K^b$-restricted $CD8^+$ T cells (DNRQYSFV and VNHRFTLV) were entirely conserved (19). Conservation was also observed for epitope FVNHRFTLV recognized by HLA-A2-restricted human $CD8^+$ T cells. A second HLA-A2-restricted human $CD8^+$ T-cell epitope (WVFPESISPV) had a single amino acid substitution (which is underlined in the sequence) in the *clone 9* gene product (WVFPEGISPV). Whether this single amino acid substitution interferes with HLA-A2-restricted CTL recognition will be important to be determined. Also relevant is

TABLE 3. NO secretion by spleen cells of DNA-immunized mice upon in vitro restimulation with the recombinant protein His-65kDa

Plasmid[a]	Nitrite concn (μM)[b]					
	Expt 1		Expt 2		Expt 3	
	Medium	His-65kDa	Medium	His-65kDa	Medium	His-65kDa
pcDNA3	3.24 ± 1.37	3.37 ± 0.22[c]	2.73 ± 1.27	3.11 ± 0.58[c]	4.32 ± 1.72	4.96 ± 1.56[c]
pclone9	2.22 ± 2.21	**8.89 ± 0.48**[d]	1.65 ± 0.88	**14.54 ± 1.12**	6.86 ± 3.72	**27.11 ± 1.16**
pSPclone9	4.19 ± 1.82	**29.02 ± 1.64**	1.46 ± 1.15	**8.25 ± 0.67**	NT[e]	NT
pIgSPclone9	4.44 ± 5.56	**27.74 ± 1.05**	1.84 ± 1.15	**5.27 ± 0.11**	2.54 ± 1.77	**20.83 ± 2.66**

[a] BALB/c mice were immunized with the indicated plasmid.
[b] Two to five weeks after the last immunization, spleen cells were collected and stimulated in vitro with the recombinant protein His-65kDa at a final concentration of 1 μg/ml or not stimulated (medium). Results represent the means obtained from triplicate cultures ± SDs.
[c] Not significantly different from values in the supernatants of cells cultured with medium only ($P > 0.05$ by the Student *t* test).
[d] Values in boldface type are significantly different from values in the supernatants of cells cultured with medium only ($P < 0.01$ by the Student *t* test).
[e] NT, not tested.

TABLE 4. Presence of anti-CD4 MAb inhibits NO secretion by spleen cells of pclone9-immunized mice upon in vitro restimulation with the recombinant protein His-65kDa

Plasmid[a]	Nitrite concn (μM)[b]					
	No antigen	Rec. His-65kDa				
		No Ab	Rat IgG	Anti-CD4 MAb	Anti-CD8 MAb	Anti-CD4 and anti-CD8 MAb
pcDNA3	2.10 ± 0.49	2.58 ± 1.05	4.28 ± 0.67	3.06 ± 0.40	2.46 ± 0.08	3.06 ± 0.70
pclone 9	2.42 ± 0.45	15.44 ± 0.24[c]	22.85 ± 2.33	3.62 ± 0.72[d]	18.03 ± 2.50[e]	3.85 ± 1.12[d]

[a] BALB/c mice were immunized with the indicated plasmids.
[b] Spleen cells were stimulated in vitro with 1 μg of recombinant (Rec.) protein His-65kDa per ml in the presence or absence of 10 μg of rat IgG per ml or with an anti-CD4 or anti-CD8 MAb or anti-CD4 and anti-CD8 MAbs. The results are expressed as averages of triplicate cultures ± SDs. No Ab, no antibody.
[c] Significantly different from values in the supernatants of cells cultured in the absence of the recombinant protein His-65kDa ($P < 0.01$ by the Student t test).
[d] Significantly different from values in the supernatants of cells cultured in the presence of rat IgG ($P < 0.01$ by the Student t test).
[e] Not significantly different from values in the supernatants of cells cultured in the presence of rat IgG ($P > 0.05$ by the Student t test).

the fact that the sequence encoding the recently described peptide representing a parasite ligand for host cells (VTVT NVFLYNRPL) is entirely conserved in the *clone 9* and *asp-2* genes (21).

Using eukaryotic expression vectors harboring the *clone 9* gene, we reported that specific immune responses to *T. cruzi* could be generated after i.m. inoculation of plasmid. Three different plasmids containing the *clone 9* gene were used for immunization. The difference between the three plasmids was the presence of sequences encoding a secretory SP in plasmid pSPclone9 or pIgSPclone9. We found that the vast majority of the immunized BALB/c mice developed specific serum antibodies as detected by ELISA.

Cell-mediated immune response was estimated in vitro by the antigen-specific IFN-γ or IL-4 secretion. Upon in vitro stimulation with the recombinant protein His-65kDa, spleen cells from mice immunized with plasmid pclone9, pSPclone9, or pIgSPclone9 secreted comparable levels of IFN-γ. We also failed to observe significant differences among spleen cells of mice injected with the different plasmids in experiments using different concentrations of antigen in culture (0.1 to 10 μg/ml [data not shown]). We concluded that under the conditions tested, the presence of different SPs does not interfere with the priming or activation of IFN-γ-producing cells in vivo.

An important characteristic of DNA immunization is the ability to prime in vivo for specific $CD8^+$ T cells. Indeed, earlier studies have shown that immunization with plasmids containing *T. cruzi* genes primed $CD8^+$ T cells that were capable of lysing infected cells (19, 46) or inhibiting intracellular parasite development in vitro (30). We attempted to detect the presence of specific $CD8^+$ T cells using 14 different synthetic peptides selected for the presence of putative sites for binding to $H\text{-}2K^d$ or $H\text{-}2L^d$. Using a very sensitive assay, the ELISPOT assay, we could not detect the presence of peptide-specific

TABLE 5. ELISPOT assay for detection of peptide-specific IFN-γ-secreting cells

Peptide[b] in culture	No. of IFN-γ secreting cells/10^6 spleen cells of mice immunized with plasmid[a]:			
	pcDNA3	pclone9	pIgSPclone9	p154/13
No peptide	17.3 ± 8.3	11.3 ± 2.3	9.3 ± 6.1	9.2 ± 1.2
CYLQRPTAV	22.0 ± 0.0	25.0 ± 1.4	5.0 ± 1.4	NT[c]
PYEIVAGYI	14.0 ± 2.8	29.0 ± 9.9	9.0 ± 1.4	NT
GYVYVDGVI	21.3 ± 3.1	24.0 ± 5.3	28.7 ± 9.2	NT
LYSDGALHL	22.0 ± 2.0	22.7 ± 3.1	27.3 ± 5.8	NT
VYIVHEHNL	31.3 ± 15.2	26.7 ± 3.1	27.3 ± 2.3	NT
DYRCVHASV	28.7 ± 1.2	34.0 ± 6.0	31.3 ± 12.2	NT
TPTAGLVGF	16.7 ± 3.1	26.0 ± 5.3	22.7 ± 8.3	NT
RPNMSRHLF	12.7 ± 8.1	10.0 ± 3.5	14.0 ± 4.0	NT
YSDGALHLL	14.0 ± 0.0	34.0 ± 5.3	3.3 ± 1.1	NT
ESKSGDAPL	18.0 ± 0.0	17.0 ± 1.4	14.0 ± 0.0	NT
GSRNGNDRL	23.3 ± 11.0	20.7 ± 6.4	12.7 ± 4.2	NT
ESEPKRPNM	22.7 ± 4.2	16.7 ± 2.3	18.0 ± 6.0	NT
ESSTPTAGL	20.0 ± 8.5	17.0 ± 1.4	12.0 ± 2.8	NT
VSWGEPKSL	16.0 ± 2.8	22.0 ± 2.8	11.0 ± 4.2	NT
Controls				
$TS_{359–367}$ (IYNVGQVSI)	13.3 ± 3.1	NT	NT	153.3 ± 9.5
Rec. protein His-65kDa[d]	12.0 ± 6.0	101.3 ± 10.3	114.0 ± 12.5	NT
Rec. protein TS[d]	6.0 ± 3.5	22.0 ± 8.5	3.0 ± 1.4	121.3 ± 5.8

[a] The number of IFN-γ secreting cells was determined by ELISPOT assay. BALB/c mice were immunized with the indicated plasmid as described in Materials and Methods. Two weeks after the last immunization, pooled spleen cells were collected from three mice.
[b] The final concentration of each peptide in culture was 2 μM.
[c] NT, not tested.
[d] The final concentration of each recombinant (Rec.) protein in culture was 10 μg/ml (~0.15 μM).

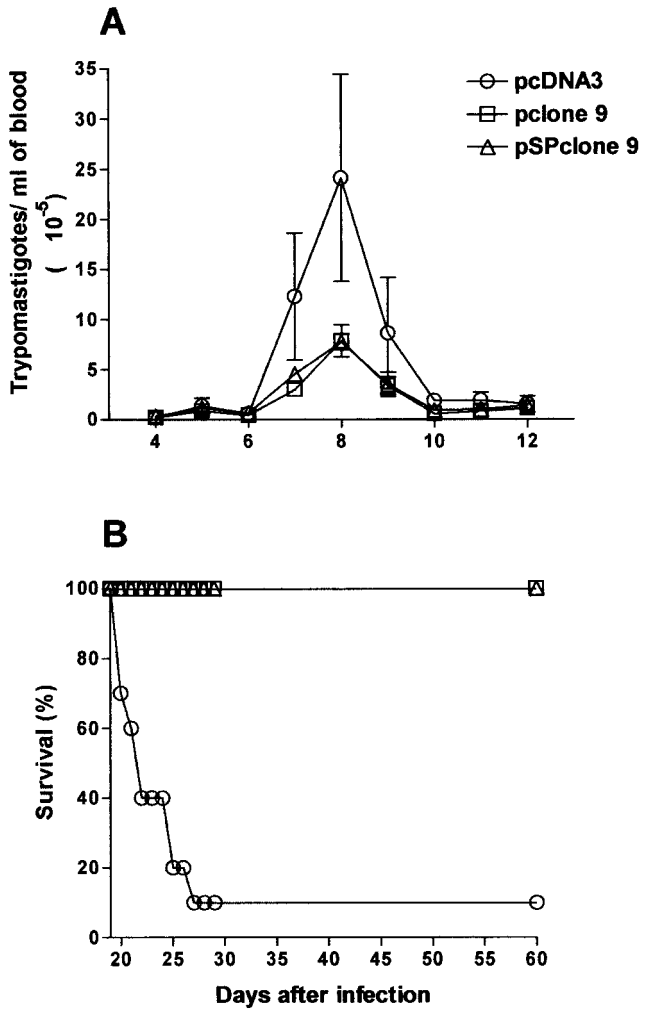

FIG. 7. Trypomastigote-induced parasitemia and mortality in BALB/c mice immunized with plasmids pclone9 and pSPclone9. BALB/c mice were immunized with pclone9, pSPclone9, or pcDNA3. Ten days after the last immunization, mice were challenged intraperitoneally with bloodstream trypomastigotes. (A) Course of infection, estimated by the number of trypomastigotes per milliliter of blood. Results are the means ± SDs obtained from mice immunized with pclone9 ($n = 12$), pSPclone9 ($n = 12$), or pcDNA3 ($n = 10$). At the peak of infection (day 8), the numbers of parasites in mice immunized with the different plasmids were compared by one-way ANOVA and Tukey HSD tests. In mice immunized with plasmid pclone9 or pSPclone9, we observed a significant reduction in the peak number of parasites from the number in animals immunized with pcDNA3 plasmid ($P < 0.0001$ by one-way ANOVA). There was no statistical difference between the numbers of parasites observed in mice immunized with pclone9 versus pSPclone9 ($P > 0.05$ by Tukey HSD test). (B) Kaplan-Meier curves for survival of mice immunized with pclone9 ($n = 12$), pSPclone9 ($n = 12$), or pcDNA3 ($n = 10$). The survival times for mice immunized with pclone9 or pSPclone9 were significantly different from that of mice immunized with pcDNA3 (control) ($P < 0.0001$ by log-rank test).

IFN-γ-secreting cells in the spleens of mice immunized with plasmid pclone9 or pIgSPclone9. This negative result could not be attributed to the ELISPOT assay, because we could easily and reproducibly detect the presence of IFN-γ-secreting cells specific for the CD8 T-cell epitope $TS_{359-367}$ (IYNVGQVSI) in the spleens of mice immunized with plasmid p154/13 (30). Nevertheless, we cannot completely exclude the possibility that the putative epitope(s) has sites for binding to $H\text{-}2K^d$, $H\text{-}2D^d$, or $H\text{-}2L^d$ distinct from the sites currently known for other antigens.

The fact that immunization of BALB/c mice with eukaryotic expression plasmids containing the *clone 9* gene elicited specific immune responses allowed us to determine whether DNA immunization with plasmid pclone9 or pSPclone9 could provide protective immunity against experimental infection. We found that immunizing the mice with these plasmids significantly reduced the peak parasite levels and that none of these mice died. In the experiments performed, 100% of the mice immunized with plasmid pclone9 or pSPclone9 survived a lethal challenge with bloodstream trypomastigotes. In contrast, 94.4% of the mice immunized with the control pcDNA3 plasmid died after challenge.

Our data on the protective immune response elicited by DNA immunization with the *clone 9* gene confirms and extends the results of the recently published study by Garg and Tarleton (10). Their study reported that immunization of C57BL/6 mice with a plasmid harboring the *asp-2* gene elicited specific antibodies and CD8 T cells (10). Immunization with the *asp-2* gene provided protective immunity against a lethal challenge with bloodstream forms of *T. cruzi* (Brazil strain), as estimated by the reduction of acute parasitemia and mortality. Also important was the observation that protective immunity induced by genetic vaccination with the *asp-2* gene reduced the skeletal muscle inflammation observed in the chronic phase of infection (10). Together, both studies emphasize the importance of the immune response to ASP-2 for the development of protective immunity to *T. cruzi* infection.

Although the protective mechanisms mediated in vivo by immunization with plasmids containing the *clone 9* gene were not addressed in the present study, it is plausible that IFN-γ-secreting T cells have a role in protection. Solid previous work established that T cells and IFN-γ participate in the control of parasitemia and mortality during the natural course of *T. cruzi* infection in mice (13, 18, 32, 40, 42; reviewed in reference 43). In addition to secreting IFN-γ, spleen cells from mice immunized with plasmids containing the *clone 9* gene also produced NO, a potent inhibitor of *T. cruzi* development in vivo (22, 24, 31, 44). Our results are compatible with those of recent studies showing that a strong Th1, but not Th2, immune response can provide a high degree of systemic immunity against experimental *T. cruzi* infection in BALB/c mice (11, 12, 37). However, the precise role of these IFN-γ-secreting $CD4^+$ T cells in protective immunity remains to be determined. Until now, there has been no clear evidence that specific $CD4^+$ Th1 cells alone can provide protective immunity against *T. cruzi* infection (11, 12, 37). These cells most likely have to interact with other T- and B-cell subsets in order to provide optimal protection against systemic infection. Future studies will be required to elucidate the effector immune mechanisms involved during the protective immune response induced by genetic vaccination with the *clone 9* gene.

It seems less likely that antibodies to the ASP-2 antigen participate in protective immunity. Amastigotes are predominantly intracellular and have never been described as targets of protective antibodies. On the other hand, extracellular trypo-

mastigotes, which are classically described as targets for protective antibodies, were not recognized by antibodies from mice immunized with plasmids containing the *clone 9* gene (Fig. 6C and D).

In summary, our results confirm and extend the results of earlier studies obtained by different research groups showing that DNA immunization can be another way to induce protective immune responses against experimental *T. cruzi* infection. The fact that DNA immunization with cDNA from amastigotes is a feasible strategy to induce immunity against *T. cruzi* may greatly facilitate future studies on immunity to these forms of the parasite using individual genes or cDNA libraries. Studies on the immune response to amastigote antigens can provide relevant basic knowledge on the complex immunopathology of Chagas' disease and may be of importance for vaccine development.

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