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DNA-Based Immunization with *Trypanosoma cruzi* Complement Regulatory Protein Elicits Complement Lytic Antibodies and Confers Protection against *Trypanosoma cruzi* Infection

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A complement regulatory protein (CRP) of *Trypanosoma cruzi* was evaluated as a vaccine candidate in a murine model of experimental *T. cruzi* infection. Recombinant CRP derived from an *Escherichia coli* expression system and a plasmid encoding the full-length *crp* structural gene under the control of a eukaryotic promoter were used to immunize BALB/c mice. Immunization with both protein and DNA vaccines resulted in a Th1-type T-cell response, comparable antibody titers, and similar immunoglobulin G isotype profiles. Only mice immunized with the *crp* DNA plasmid produced antibodies capable of lysing the parasites in the presence of complement and were protected against a lethal challenge with *T. cruzi* trypomastigotes. These results demonstrate the superiority of DNA immunization over protein immunization with the recombinant CRP. The work also supports the further investigation of CRP as a component of a multigene, anti-*T. cruzi* DNA vaccine.

The protozoan parasite *Trypanosoma cruzi* causes a persistent infection which can lead to Chagas' disease, a major public health concern in Latin America, where an estimated 18 million people are infected (23). The infection results in a generally self-limiting acute parasitemic phase, followed by an indeterminant phase where parasitemia is commonly undetectable and most patients remain asymptomatic. Approximately 30% of individuals in the indeterminant phase progress to a chronic, symptomatic phase involving severe cardiomyopathy or gastrointestinal pathology. Several recent studies have provided information regarding the protective roles of antibodies, Th1-type cytokines, and cytotoxic T cells (CTL) (4, 8, 20). An increased understanding of the host immune responses to the organism and the pathogenesis of the disease (20) has provided a rationale for the pursuit of vaccine development as a means of control of *T. cruzi* infections. Recent advances in DNA vaccine technology make this an attractive vehicle for vaccine development against Chagas' disease for immunologic as well as economic reasons.

The complement regulatory protein (CRP) of *T. cruzi* has been described as a virulence factor that enables the parasite to escape lysis by the host complement system (14). The expression of the *crp* gene is developmentally regulated and is coincident with conversion of the parasite from the complement-sensitive insect stage (epimastigote) to the complement-resistant bloodstream stage (trypomastigote) (14, 17). The CRP is a surface glycoprotein that interferes with complement activation via noncovalent binding to the complement proteins C3b and C4b, the central components of the C3 convertase of the alternative and classical complement pathways, respectively (12, 16). Antibodies that block the complement regulatory activity of the CRP promote complement-mediated lysis of the parasites and have been detected in sera from chagasic patients (15). Transfection of the insect-stage epimastigotes with the *crp* gene resulted in constitutive expression of *crp* and conversion of the complement-sensitive epimastigotes to a complement-resistant phenotype (14). These results suggest that the CRP is necessary and sufficient to confer complement resistance to the parasites. Inasmuch as the CRP represents a virulence factor important in the survival of the extracellular stage of *T. cruzi*, we investigated the utility of the CRP in vaccination studies in a murine model of acute *T. cruzi* infection.

We report here a comparison of humoral and cellular anti-CRP responses in recombinant protein and DNA-based immunizations. Mice immunized with purified, recombinant CRP or naked DNA encoding the *crp* gene were subjected to a challenge with *T. cruzi* trypomastigotes, and survival and parasitemia levels were compared to those for the infected control mice. Our findings show that although titers of CRP-specific antibodies, immunoglobulin G (IgG) isotype profiles, and production of gamma interferon (IFN-γ) were similar or higher in protein-immunized animals compared to DNA-immunized mice, only the *crp* DNA-immunized mice were able to produce antibodies that supported complement-mediated lysis of the parasite. Indeed, when immunized mice were challenged with *T. cruzi* trypomastigotes, the *crp* DNA-immunized animals were protected, whereas no protection was observed in protein-immunized animals.

MATERIALS AND METHODS

Mice and parasites. C3H/HeJ mice were bred at the Pasteur Institute. BALB/c mice, 8 to 10 weeks old, were obtained from Iffa Credo (l'Arbresle, France) and from Jackson Laboratory (Bar Harbor, Maine). Female mice were used for naked DNA immunizations. Tissue culture-derived parasites (Y strain) were passed weekly in naïve BALB/c mice or cultured on NIH 3T3 fibroblasts as described elsewhere (17). Parasitemia was determined at the tail vein by optical microscopy (1).

Plasmid construction and antigens. A full-length cDNA encoding the *T. cruzi* CRP was isolated by reverse transcription-PCR as previously described (17). The *T. cruzi crp* cDNA encoding the mature protein (starting at nucleotide 303) was subcloned into the pTrcHis expression vector (Invitrogen), and recombinant histidine-tagged CRP (CRP-His) was purified from *Escherichia coli* by Ni^{2+} chelation chromatography as previously described (17). The eukaryotic expression vector pBC12BI (3) was obtained from the American Type Culture Collection. The pBC12BI.*crp* construct was generated as a translational fusion that

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included the translation initiation codon and first six codons of the rat preproinsulin gene and coding sequence for amino acids 7 through 1003 of the *crp* cDNA. pBC12BI.*crp-daf* was generated by exchanging the regions of the CRP carboxy-terminal domain for glycosylphosphatidylinositol (GPI) anchor addition and the GPI anchor addition domain of human decay-accelerating factor (DAF) cDNA (M. Beucher, A. Mascilli, and K. A. Norris, unpublished data). Specifically, coding sequences for amino acids 953 to 979 of CRP were replaced with coding sequences for amino acids 317 to 347 of DAF (numbering begins at the first amino acid of the mature proteins). Details of the construction and cloning will be presented elsewhere. Plasmid DNA was purified by anion-exchange chromatography using Qiagen maxi kits. DNA used for immunizations was sterilized by ethanol precipitation and resuspended in lipopolysaccharide-free phosphate-buffered saline (PBS) (GIBCO, Grand Island, N.Y.).

In vitro transfection with pBC12BI.*crp-daf* expression vector. COS-K1 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 5 mM L-glutamine, 0.2 mM sodium pyruvate, and 10% fetal calf serum, all from GIBCO. Cells were seeded at 3×10^5 cell per ml in six-well Costar plates and incubated overnight at 37°C in 5% CO_2 prior to transfection. Cells were transfected with 0.5 to 2.0 μg of pBC12BI.*crp-daf* DNA or vector alone and 6 μl of Lipofectamine (GIBCO) as directed by the manufacturer. Forty-eight hours posttransfection, the cells were washed once in Tris-buffered saline (50 mM Tris [pH 7.6], 150 mM NaCl), lysed in 250 μl of Tris-buffered saline containing 1% Nonidet P-40 (Pierce), and incubated on ice for 30 min. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described below.

Immunization and *T. cruzi* challenge of mice. BALB/c and C3H/HeJ mice were immunized by intraperitoneal injection of 15 μg of recombinant CRP-His emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) and boosted twice with 30 μg of recombinant CRP-His emulsified in Freund's incomplete adjuvant (Sigma, St. Louis, Mo.) every 2 weeks. Additional mice were injected with Freund's complete adjuvant and boosted with Freund's incomplete adjuvant. For DNA-based immunizations, 100 μg of pBC12BI.*crp-daf* DNA or vector DNA was dissolved in 50 μl of PBS and injected intramuscularly in the tibialis anterior muscles of mice that had been briefly anesthetized by metaphane inhalation. BALB/c mice immunized with DNA or recombinant protein were challenged intravenously (i.v.) 2 weeks after the last boost with 2×10^6 *T. cruzi* strain Y trypomastigotes. Parasitemia was monitored by microscopic enumeration of motile parasites in tail blood, and mortality was recorded daily.

Enzyme-linked immunosorbent assay (ELISA) and isotype determination. Microtiter plates (Nunc Immunoplates, Nunc, Roskilde, Denmark) were coated overnight at 4°C with CRP-His (1 μg/ml) in 50 μl of PBS. Plates were washed three times with washing buffer (PBS [pH 7.4] containing 0.1% Tween 20) and then incubated with blocking buffer (PBS containing 1% gelatin) for 2 h at room temperature. Mouse polyclonal sera were diluted in blocking buffer, added to duplicate series of wells, and incubated for 2 h at room temperature. Wells were washed five times with washing buffer, and then 50 μl of peroxidase-labeled goat anti-mouse total IgG serum (Southern Biotechnology, Birmingham, Ala.) diluted 1:2,000 was dispensed into each well. The coloration was developed by adding 50 μl of ABTS [2,2-azinobis(3-ethylbenzthiazoline sulfonic acid)] peroxidase substrate solution (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) and incubating at the mixture 37°C for 10 min. The color intensity was determined at optical densities (OD) of 405 and 650 nm with a double-length automated plate reader (Molecular Devices, Medi-Sciences).

For antibody isotyping, peroxidase-conjugated second antibodies against IgG1 (diluted 1:2,000), IgG2a (1:2,000), IgG2b (1:2,000), IgG3 (1:1,000), and IgM (1:2000) (Southern Biotechnology) were added after incubation with polyclonal mouse sera and then incubated for 1 h at room temperature (RT). Plates were washed five times, and the coloration was revealed as described above. Positive levels of specific isotypes were considered with a value (V) equivalent to at least the mean value (V_0) obtained from normal control sera (OD_0) plus three times the standard deviation (SD) ($V_0 = OD_0 + 3 \times SD$; ratio = V/V_0; positive if ratio >1). ELISA arbitrary units (EU) were calculated by the difference between the mean values of CRP-His and three times the mean value obtained for normal control sera according to the following equation: $EU = (OD_{CRP\text{-}His} - 3 \times OD_0)$.

SDS-PAGE and immunoblotting. SDS-PAGE was performed as described by Laemmli (9), using 8.5% polyacrylamide gels (Bio-Rad, Richmond, Calif.). Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose in 50 mM Tris base–380 mM glycine–0.1% SDS–20% methanol at 30 V for 16 h. Filters were blocked with 5% nonfat dry milk in PBS and then probed with test or normal mouse sera at dilutions indicated in the text. The filters were counterprobed with horseradish peroxidase-labeled goat anti-mouse antibodies at 1:100,000 dilution. Filters were developed using Supersignal West Femto maximum sensitivity substrate (Pierce Chemical Co.).

Cytokine assays. Spleen cells from either protein- or DNA-immunized mice were harvested 10 days after the last immunization. Cells from individual mice were resuspended in RPMI 1640 medium with Glutamax-I (GibcoBRL, Life Technologies) supplemented with 10% fetal calf serum and antibiotics (1% penicillin-streptomycin). Cells were adjusted to a concentration of 2.5×10^5 cells/ml and were cultured in 24-well plates (Costar) for 48 h alone or with added recombinant CRP-His (2 to 10 μg/ml) or concanavalin A (ConA; 5 μg/ml) as a mitogenic reagent. Supernatants were harvested at 72 h for cytokine assay. IFN-γ, interleukin-2 (IL-2), IL-10, and IL-4 were measured by ELISA using monoclonal antibodies (MAbs) as previously described (10), with modifications. Briefly, serial dilutions of known amounts of specific standards and pure cell culture supernatants were incubated for 2 h in 96-well plates (Nunc) that had previously been coated with unlabeled specific anti-interleukin antibodies diluted in PBS and incubated overnight at 4°C. Second-step reactions were done by incubating the washed plates with a biotin-labeled anticytokine MAb directed against a different epitope of the cytokine under test for 1 h at RT. After further washings, the plates were incubated with streptavidin-peroxidase for 1 h at RT. Following the addition of the substrate, the absorbance at 490 and 650 nm was determined. Concentrations were expressed as picograms per milliliter calculated according to calibration curves made with serial dilutions of recombinant murine cytokines. The detection limits of the assays were 40 pg/ml for IFN-γ, 40 pg/ml for IL-2, 100 pg/ml for IL-10, and 30 pg/ml for IL-4. The rat anti-mouse cytokine MAbs used were anti-IFN-γ (clones R46A2 and AN-18), anti-IL-2 (clones JES1A12 and JES5H4), anti-IL-4 (clones BDB4-1D11 and BVD6-24G2), and anti-IL-10 (clones JES5 and SXC-1). Clones producing anti-IFN-γ, anti-IL-2, anti-IL-4, and anti-IL-10 were obtained from DNAX Research, Palo Alto, Calif. and provided by P. Minoprio. The cytokines used were recombinant IFN-γ and IL-2 (Pharmingen), supernatant from the D10 cell line (a Th2 clone) for IL-4, and supernatant from the J558-10 cell line (hybridoma transfected with the IL-10 gene) for IL-10.

Complement-mediated lysis assay. Tissue culture-derived *T. cruzi* trypomastigotes were washed once in PBS–1% glucose and resuspended at 2×10^7/ml. A 25-μl portion of this suspension was incubated with an equal volume of either normal mouse sera or test serum diluted 1:2 for 1 h at 37°C. The cells were washed once (1,300 × *g*, 10 min, 4°C) and resuspended in 100 μl of rabbit complement diluted 1:4 (Low Tox M; Cedarlane) for an additional hour at 37°C. Control tubes included parasites incubated with test antiserum and heat-inactivated complement (56°C, 30 min) and parasites incubated with preimmune sera and complement. In addition, rabbit anti-CRP IgG fraction (5 mg/ml) was used as a positive control for lysis (13). Preimmune and postimmune pooled sera were used in these assays. Samples were assayed in triplicate, and the number of motile trypomastigotes was determined in a hemocytometer. Percent lysis was determined as 100 − (number of parasites after incubation with rabbit complement/number of parasites after treatment with heat-inactivated complement) × 100.

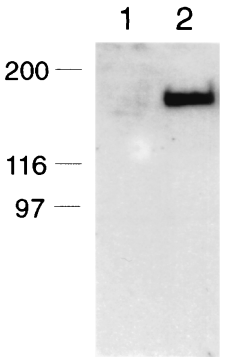

FIG. 1. Western blot analysis of COS-K1 cells transiently transfected with pBC12BI plasmid DNA (lane 1) or pBC12BI.*crp-daf* plasmid DNA (lane 2). Detergent-solubilized lysates of transfected cells (5 μg of total protein) were separated by SDS-PAGE and immunoblotted with anti-CRP antiserum (1:20,000). Molecular masses are indicated at the left in kilodaltons.

RESULTS

Expression of CRP protein in vitro. We have determined that the GPI anchor addition signal sequence of the *T. cruzi crp* gene is not efficiently recognized in mammalian cells, resulting in a poor overall expression level and failure of the recombinant CRP to reach the cell surface (Beucher et al., unpublished). To enhance the efficiency of proper posttranslational modification of CRP and surface expression in COS cells, it was necessary to replace the carboxy-terminal GPI signal sequence of the *crp* gene with the corresponding sequence from DAF. The mature protein produced in COS cells upon transfection with plasmid pBC12BI.*crp-daf* is of the expected molecular mass (Fig. 1) and is anchored to the cell surface by a GPI linkage as determined by release of the CRP from cells after treatment with phosphatidylinositol-specific phospholipase C; the *crp-daf* gene fusion was designed such that no

daf-derived amino acids are produced in the recombinant protein (Beucher et al., unpublished). No CRP was detected in cells transfected with the vector plasmid, pBC12BI (Fig. 1, lane 1).

Induction of anti-CRP humoral responses in mice immunized with recombinant CRP or *crp* DNA. To investigate the humoral response against CRP, C3H/HeJ and BALB/c mice were immunized intraperitoneally with CRP-His recombinant protein in Freund's adjuvant or intramuscularly with pBC12BI.*crp-daf* in PBS. When DNA-based immunizations were performed, antibodies were initially detected 1 week after the priming injection, and antibodies increased with subsequent boosts (Fig. 2A). In contrast, anti-CRP antibodies were detectable in the CRP-His-immunized mice only after three injections of recombinant protein but eventually reached the levels of the DNA-immunized mice. By day 60 after the priming injections, both immunization protocols resulted in anti-CRP antibody titers in excess of 1:10,000 (Fig. 2B). This high anti-CRP antibody titer was observed in BALB/c and C3H/HeJ mice and is significantly greater than that observed in mice experimentally infected with *T. cruzi*, where the anti-CRP titer is generally less than 1:1,000 (data not shown).

Analysis of immune sera from CRP-His- and pBC12BI.*crp-daf*-immunized mice revealed a predominant production of IgG, with IgM minimally detectable. The EU for IgM from at least two series of immunization with either protein or DNA was less than 0.01 (not shown). Isotyping of sera from pBC12BI.*crp-daf*-immunized mice showed that all mice analyzed were able to specifically produce IgG1, IgG2a, and IgG2b isotypes but minimally detectable IgG3. Similar results were found when analyzing sera from CRP-His-immunized mice, although levels of specific IgG1 were reproducibly higher (Fig. 2C). Interestingly, although titer and isotype profiles of the antibodies from protein-immunized mice were similar to those from DNA-based immunizations, antibodies from pBC12BI.*crp-daf*-immunized mice were capable of complement-mediated killing of trypomastigotes in vitro, whereas minimal killing was observed when parasites were treated with anti-CRP-His antibodies and complement (Fig. 3). In these experiments, pooled sera from DNA- or protein-immunized mice were tested for the ability to support complement-mediated lysis of trypomastigotes. Specific lysis of parasites could not be detected in repeated assays with sera from CRP-His-immunized mice compared with preimmune sera. In contrast, treatment of parasites with sera from pBC12BI.*crp-daf*-immunized mice consistently resulted in greater than 60% lysis. Results of a representative experiment are shown in Fig. 3.

Cytokine analysis of spleen cells from CRP-His- and *crp* DNA-immunized spleen cells. To analyze whether spleen cells from either pBC12BI.*crp-daf*- or CRP-His-immunized mice were able to secrete IFN-γ, IL-2, IL-4, or IL-10 after stimulation with CRP-His antigen, an ELISA for each specific cytokine was performed. As shown in Fig. 4, IFN-γ production was observed in both cases after stimulation with various antigen concentrations, and the levels were higher in cultured supernatants from CRP-His-immunized mice spleen cells (Fig. 4B). No significant levels were detected for the other tested cytokines (data not shown). Spleen cells from all experimental and control groups proliferated to comparable levels in response to treatment with the mitogen, ConA (not shown).

Immunization with pBC12BI.*crp-daf* DNA protects mice from a lethal *T. cruzi* challenge. Inasmuch as both DNA- and recombinant protein-based immunization strategies generated significant anti-CRP immune responses in mice, we next tested whether these immunization protocols could afford protection against an experimental *T. cruzi* infection. BALB/c mice were

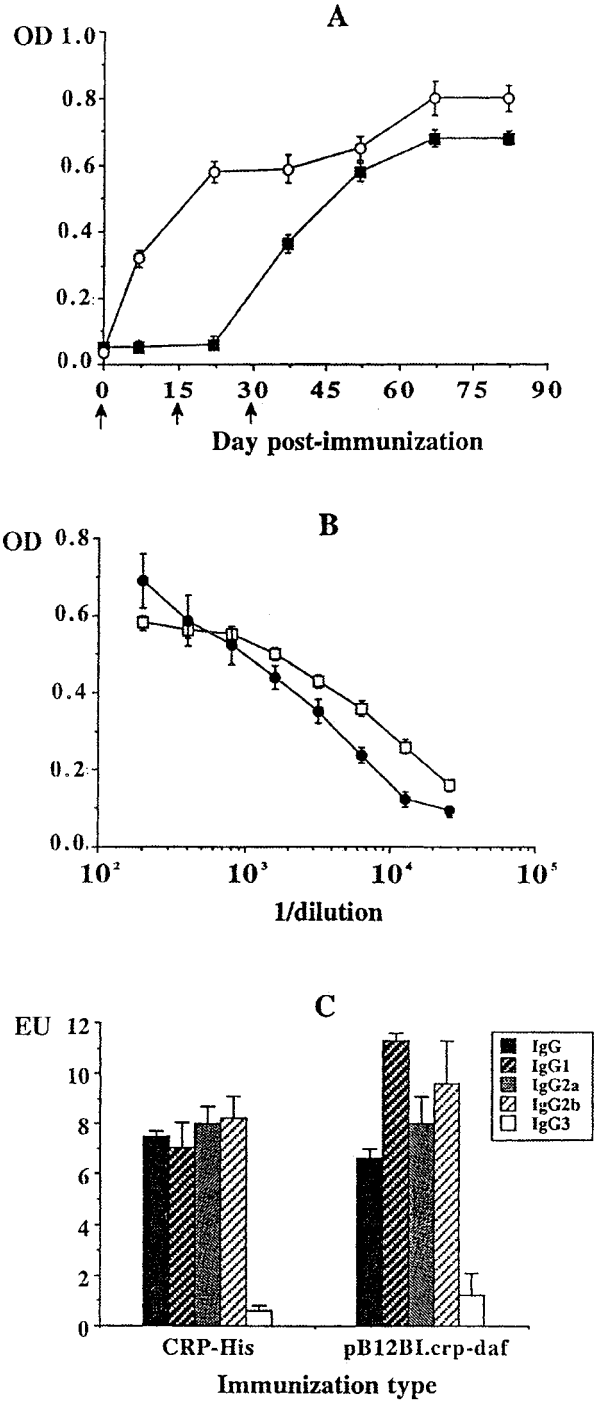

FIG. 2. Analysis of anti-CRP humoral responses in mice immunized with recombinant CRP-His or pBC12BI.*crp-daf* plasmid DNA. (A) Kinetics of antibody production in C3H/Hej mice immunized with CRP-His or pBC12BI.*crp-daf* plasmid DNA. Sera were taken 10 days after each dose from mice immunized with pBC12BI.*crp-daf*, pBC12BI, CRP-His, or incomplete Freund's adjuvant and analyzed by ELISA for antibodies to recombinant CRP-His. Values were considered positive when greater than three times the mean values for adjuvant or control mice in each case (EU = ODtest/3 × ODcontrol). (B) Titration of sera from CRP-His- or pBC12BI.*crp-daf*-immunized mice taken 10 days after the last boost. Serial dilutions were performed for each serum sample, and the mean OD at each dilution is reported. (C) IgG isotypes of anti-CRP antibodies from pBC12BI.*crp-daf*- or CRP-His-immunized mice. Total IgG and specific isotypes were quantified 10 days after the last boost by testing on ELISA plates coated with recombinant CRP-His (1 μg/ml). Serum samples from 5 to 10 CRP-His- or pBC12BI.*crp-daf*-immunized mice were diluted 1:100. Results are expressed in EU (mean OD/3 × mean $OD_{control}$) ± SD. All assays were done in triplicate, and the results presented are from a representative experiment.

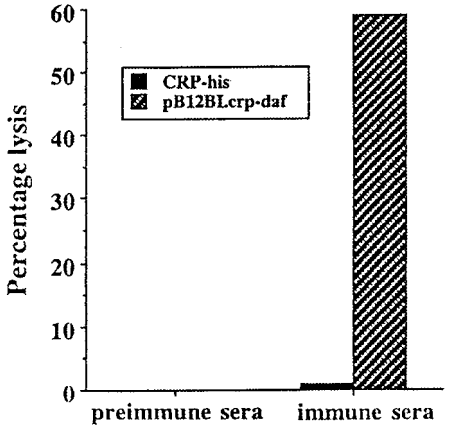

FIG. 3. Complement-mediated lysis of *T. cruzi* trypomastigotes incubated with preimmune serum or anti-CRP antiserum from either CRP-His- or pBC12BI.*crp-daf* plasmid-immunized mice taken 10 days after the last boost. Parasites were treated with antisera pooled from five to eight mice, followed by treatment with rabbit complement (1:4) or heat-inactivated complement for 1 h at 37°C. Survival was scored by microscopic enumeration of motile parasites. Results are from a representative experiment, and values represent the mean and SD for triplicate samples.

immunized with pBC12BI.*crp-daf* or CRP-His as described above. Two weeks after the last injection, mice were infected i.v. with Y strain trypomastigotes. Parasitemia was monitored beginning at day 4 postinfection and peaked at day 6 in all groups. Only the pBC12BI.*crp-daf*-immunized mice showed significant reduction in peak parasitemia (Table 1) and survived the lethal challenge (Table 1 and Fig. 5B). CRP-His-immunized mice showed no significant difference in peak parasitemia or survival compared to adjuvant-injected control animals (Table 1 and Fig. 5A).

DISCUSSION

The main finding of this work is that a protective immune response to *T. cruzi* can be elicited by immunization with naked DNA encoding the *crp* structural gene. The DNA delivery system proved to be superior to immunization with purified, *E. coli*-derived recombinant CRP protein with respect to protection from a lethal challenge and reduction in peak parasitemia levels. Although both immunization strategies resulted in high-titer anti-CRP antibody production of similar isotype profiles, only DNA immunization resulted in the production of antibodies that could lyse parasites in conjunction with complement and protect them from a lethal infection. The generation of complement lytic antibodies is of interest because it was previously reported that lytic antibodies were associated with protection from infection and were elicited following active *T. cruzi* infection and not with conventional immunogens, such as heat-killed trypomastigotes or cell extracts (5–7). It has been suggested that the antigens responsible for eliciting lytic antibodies were either labile or secreted from live parasites, since heat-killed or fixed parasites did not induce such antibodies. We have previously shown that immunization with the CRP purified from *T. cruzi* trypomastigotes was sufficient to generate lytic antibodies (13). It was proposed that antibodies to the CRP that specifically neutralize the complement regulatory activity would support lysis, whereas nonneutralizing, anti-CRP antibodies would not be lytic (12). The recombinant CRP-His protein purified from *E. coli* has no detectable complement regulatory activity as determined by in vitro complement inhibitory assays or by C3b binding assays, presumably due to lack of proper conformation and/or glycosylation (K. Norris, unpublished data). The results support the hypothesis that antibodies produced by immunization with the nonnative, *E. coli*-derived recombinant CRP could not neutralize the activity of the native CRP produced by the parasites and thus did not support complement-mediated lysis of trypomastigotes. Indeed, although these animals had high-titer anti-CRP antibodies, they were not protected from lethal infection. We have found that transfection of mammalian cells with pBC12BI.*crp-daf* produces functional CRP capable of restricting complement activation (Beucher et al., unpublished). It is therefore likely that the anti-CRP neutralizing antibodies induced by DNA immunization are directed to conformationally constrained epitopes present in the native, parasite-derived protein and in the functional, recombinant CRP produced in mammalian expression systems but not in the *E. coli*-derived, nonnative CRP. This difference in complement lytic activity of the antibodies from DNA- and protein-immunized animals is not due to different antibody isotype profiles, since there was essentially no difference in the isotypes generated by either immunization strategy. In contrast to the antibodies to CRP-His, the anti-CRP antibodies from DNA-immunized mice could support complement-mediated lysis of parasites in vitro

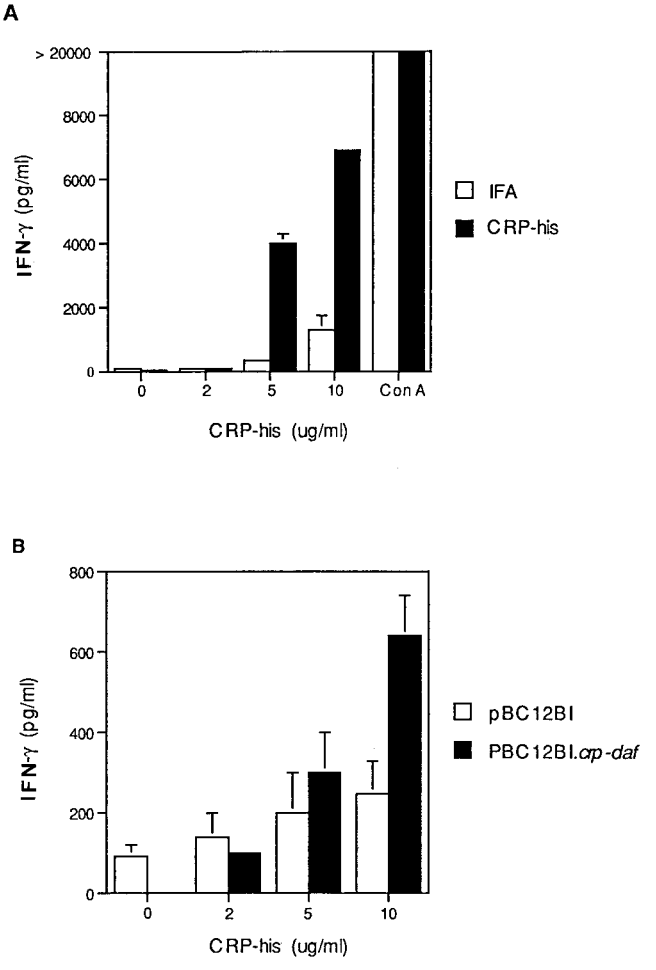

FIG. 4. IFN-γ production by spleen cells from pBC12BI.*crp-daf*- or CRP-His-immunized mice. Cells were unstimulated or stimulated with CRP-His (2 to 10 μg/ml) or ConA (5 μg/ml). Data are expressed as the mean ± SD for three animals in each group. IFA, incomplete Freund's adjuvant.

TABLE 1. Lethal *T. cruzi* challenge of DNA- or recombinant protein-immunized BALB/c mice[a]

Immunogen	No. of survivors/ no. challenged	% Survival[b]	Parasitemia on day 6 (10^5)[c]
pBC12BI.*crp-daf*	5/5		5.8 ± 2.5
	5/5		3.9 ± 3.1
		100	
pBC12BI	1/5		28.1 ± 4.9
	0/5		30 ± 18.7
		10	
CRP-His	0/6		38.3 ± 16.4
	0/5		ND[d]
		0	
Adjuvant-PBS	0/3		35.3 ± 11.4
	0/3		ND
		0	

[a] Mice were immunized with DNA plasmids or purified recombinant CRP-His as described in Materials and Methods. Two weeks after the final boost, mice were challenged i.v. with 2×10^6 *T. cruzi* Y strain trypomastigotes.

[b] Determined at 40 days postinfection. Significant protection against lethal *T. cruzi* challenge was observed in mice immunized with pBC12BI.*crp-daf* compared with animals receiving vector alone (one-sided $P < 0.0002$, Wilcoxon two-sample rank sum test).

[c] Mean parasitemia levels ± SD for three to six mice per experiment. The peak parasitemia level (day 6 postinfection) of pBC12BI.*crp-daf*-immunized mice was significantly lower than that of control animals ($P < 0.0001$, $t = 5.65$, Student's *t* test). No significant difference in survival or mean peak parasitemia values was observed in CRP-His-immunized mice compared to adjuvant controls.

[d] ND, not determined.

and may therefore contribute to the overall protective immune response observed in these mice.

DNA-based immunization strategies have been used successfully to elicit both humoral and cellular responses and have been particularly useful in inducing cytotoxic T cells (3). As CTL have been shown to have a prominent role in controlling *T. cruzi* infection (18, 19), it is also possible that difference in protection observed between protein- and DNA-immunized mice in this study may be due to the failure of the protein immunization protocol to elicit CTL.

One of the strengths of the DNA-based immunization strategy is also reflected in the very high titers of antibodies that are readily achieved. In these experiments, anti-CRP antibodies were detectable after the priming dose of pBC12BI.*crp-daf* DNA, whereas protein immunization required three injections before anti-CRP antibodies could be detected. In addition, after three or four immunizing doses, the anti-CRP titer of sera from DNA-immunized mice was in excess of 1:10,000, whereas anti-CRP titer in sera from mice chronically infected with the Y strain of *T. cruzi* is generally less than 1:1,000. These results suggest that the present DNA construct, which directs expression to the cell surface in in vitro expression experiments (Beucher et al., unpublished), is an excellent stimulator of the humoral response to CRP, and antibody response by immunization exceeds that produced during the course of infection.

An investigation of the cytokine profile of T cells recovered from DNA- and protein-immunized mice revealed that both immunization protocols generated antigen-specific IFN-γ-producing cells. These results suggest that the immunization protocols used were capable of eliciting CRP-specific $CD4^+$ T helper cells, since the assay conditions used would be unlikely to stimulate CTL or NK cells, two other IFN-γ-producing cell types. No IL-10 or IL-4 was detected in these experiments, suggesting that a predominantly Th1-type response was elicited by both immunization strategies. Interestingly, although the level of IFN-γ produced in these assays was higher in protein-immunized mice than in DNA-immunized mice, only the DNA-immunized mice were protected against *T. cruzi* infection.

DNA-based immunization protocols have been shown to produce a range of immune responses and will likely be an effective strategy of vaccine development for many infectious diseases. DNA immunization strategies will be particularly useful for infectious diseases prevalent in developing countries because of the ease with which they can be manipulated, the relatively low cost, and their ease of delivery. The possibility of successful vaccine development in Chagas' disease has been strengthened by recent advances in our understanding of the nature of protective immunity to *T. cruzi* and the pathogenesis of disease (19–21). In addition, other molecularly defined antigens have been shown to be protective in both DNA-based and protein-based immunization strategies (2, 11, 22). The results presented here demonstrate that DNA-based immunization with the *T. cruzi* CRP may be important to a multicomponent vaccine, particularly with respect to generating a neutralizing humoral response.

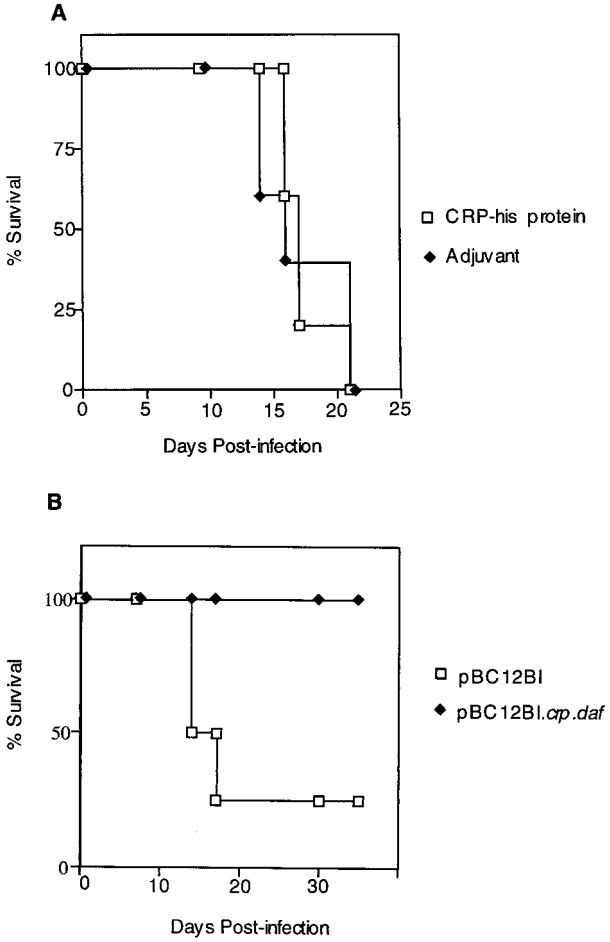

FIG. 5. *T. cruzi* challenge of BALB/c mice immunized with pBC12BI.*crp-daf* plasmid DNA or recombinant CRP-His. Mice were immunized with either CRP-His or a PBS-adjuvant emulsion (A) or pBC12BI.*crp-daf* or pBC12BI plasmid DNA (B) as described in Materials and Methods. Two weeks following the last injection, mice were challenged i.v. with 2×10^6 *T. cruzi* trypomastigotes, and survival was monitored daily for 40 days.

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